

UTILITY PATENT APPLICATION TRANSMITTAL

For new nonprovisional applications under 37 CFR 1.53(b)

Attorney Docket No.

0660-0135-0X CIP

First Named Inventor

Shirley LONGACRE-ANDRE

Title

RECOMBINANT PROTEIN CONTAINING A C-
TERMINAL FRAGMENT OF PLASMODIUM MSP-1

APPLICATION ELEMENTS

USPTO Form 361, 1997
MPEP chapter 600 concerning utility application
contents

ADDRESS TO:
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Patents
Box Patent Applications
Washington, DC 20231

1. ☒ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)

2. ☒ Specification [Total Pages 70]

3. ☒ Drawing(s) [Total Sheets 59]

4. ☒ Oath or Declaration [Total Pages]

a. ☐ Newly executed (original or copy)

b. ☐ Copy from a prior application (37 CFR
1.63(d))

(for continuation/divisional with Box 17 completed)
[Note Box 5 below]

i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting
inventor(s) named in the prior
application,
see 37 CFR 1.63(d)(2) and 1.33(b).

5. ☐ Incorporation By Reference (useable if Box 4b is
checked)

The entire disclosure of the prior application, from
which a copy of the oath or declaration is supplied
under Box 4b, is considered as being part of the
disclosure of the accompanying application and is
hereby incorporated by reference therein.

ACCOMPANYING APPLICATION PARTS

6. ☐ Assignment Papers (cover sheet & document(s))

7. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)

8. ☐ English Translation Document (if applicable)

9. ☐ Information Disclosure ☐ Copies of IDS Citations
Statement (IDS)/PTO-1449

10. ☒ Preliminary Amendment

11. ☒ White Advance Serial No. Postcard

12. ☐ Small Entity ☐ Statement filed in prior app.
Statement(s) Status still proper and desired

13. ☐ Certified Copy of Priority Document(s)

14. ☒ Other: Request for Priority \$119
Request for Priority \$120
List of Inventor Names and Addresses

15. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of International Application No. PCT/FR97/00290

16. Amend the specification by inserting before the first line the sentence:

This application is a ☐ Continuation ☐ Division ☒ Continuation-in-part (CIP)
of International Application No. PCT/FR97/00290, filed on February 14, 1997.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): Shirley LONGACRE-ANDRE et al.

Serial No: New U.S. Application

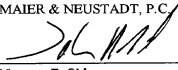
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Title: RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF
PLASMODIUM MSP-1**FEE TRANSMITTAL**Assistant Commissioner For Patents
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CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS	67 -20=	47	X \$22=	\$1,034.00
	INDEPENDENT	6 -3=	3	X \$82=	\$246.00
	MULTIPLE DEPENDENT CLAIMS (if applicable)			+ \$270=	\$0.00
	LATE FILING OF DECLARATION			+ \$130=	\$130.00
	BASIC FEE				\$790.00
	TOTAL OF ABOVE CALCULATIONS =				\$2,200.00
	Reduction by 50% for filing by small entity				
	<input type="checkbox"/> FILING IN NON-ENGLISH LANGUAGE			+ \$130=	\$0.00
	<input type="checkbox"/> RECORDATION OF ASSIGNMENT			+ \$40=	\$0.00
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Respectfully submitted,

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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

Shirley LONGACRE-ANDRE et al. :

SERIAL NO.: New U.S. Application :

FILED: Herewith :

FOR: RECOMBINANT PROTEIN CONTAINING A
C-TERMINAL FRAGMENT OF PLASMODIUM
MSP-1

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE CLAIMS

Please amend the claims as follows:

Claim 5, line 1, delete "or claim 3".

Claim 6, line 1, replace "A" with --The--.

Claim 10, line 1, insert --recombinant-- before "protein".

Claim 11, line 1, insert --protein-- after "recombinant".

Claim 29, line 1, replace "of", first occurrence, with --comprising--.

Claims 33-48, line 1 of each, replace "vaccinating" with --vaccination--.

Claim 67, line 1, replace "vaccine" with --vaccination--.

RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF *PLASMODIUM* MSP-1

The invention relates to novel active principles for vaccines derived from the major surface protein in merozoite forms of a *Plasmodium* which is infectious for mammals, especially humans, more generally termed MSP-1.

MSP-1 has already been the subject of a number of studies. It is synthesized in the schizont stage of *Plasmodium* type parasites, in particular *Plasmodium falciparum*, and is expressed in the form of one of the major surface constituents of merozoites both in the hepatic stage and in the erythrocytic stage of malaria (1, 2, 3, 4). Because of the protein's predominant character and conservation in all known *Plasmodium* species, it has been suggested that it could be a candidate for constituting anti-malarial vaccines (5, 6).

The same is true for fragments of that protein, particularly the natural cleavage products which are observed to form, for example during invasion by the parasite into erythrocytes of the infected host. Among such cleavage products are the C-terminal fragment with a molecular weight of 42 kDa (7, 8) which is itself cleaved once more into an N-terminal fragment with a conventional apparent molecular weight of 33 kDa and into a C-terminal fragment with a conventional apparent molecular weight of 19 kDa (9) which remains normally fixed to the parasite membrane after the modifications carried out on it, via glycosylphosphatidylinositol (GPI) groups (10, 11).

It is also found at the early ring stage of the intraerythrocytic development cycle (15, 16), whereby the observation was made that the 19 kDa fragment could play a role which is not yet known, but which is doubtless essential in re-invasive processes. This formed the basis for hypotheses formed in the past that that protein could constitute a particularly effective target for possible vaccines.

C.P. Application
with 59 plates

It should be understood that the references frequently made below to the p42 and p19 proteins from a certain type of *Plasmodium* are understood to refer to the corresponding C-terminal cleavage products of the MSP-1 protein of that *Plasmodium* or, by extension, to products containing substantially the same amino acid sequences, obtained by genetic recombination or by chemical synthesis using conventional techniques, for example using the "Applied System" synthesizer, or by "Merrifield" type solid phase synthesis. For convenience, references to "recombinant p42" and "recombinant p19" refer to "p42" and "p19" obtained by techniques comprising at least one genetic engineering step.

Faced with the difficulty of obtaining large quantities of parasites for *P. falciparum* and the impossibility of cultivating *P. vivax* *in vitro*, it has become clear that the only means of producing an anti-malaria vaccine is to resort to techniques which use recombinant proteins or peptides. However, MSP-1 is very difficult to produce whole because of its large size of about 200 kDa, a fact which has led researchers to study the C-terminal portion, the (still unknown) function of which is probably the more important. In addition, the extensive polymorphism in the N-terminal portion of MSP-1 has negative implications for the use of these parts of the molecule in vaccine preparation.

Recombinant proteins concerning the C-terminal portion of the *P. falciparum* MSP-1 which have been produced and tested in the monkey (12, 40, 41) are:

- a p19 fused with a glutathione-S-transferase produced in *E. coli* (40);
- a p42 fused with a glutathione-S-transferase produced in *E. coli* (12);
- a p19 fused with a polypeptide from a tetanic anatoxin and carrying auxiliary T cell epitopes produced in *S. cerevisiae* (12);
- a p42 produced in a baculovirus system (41).

A composition containing a p19 protein fused with a glutathione-S-transferase produced in *E. coli* combined with alum or liposomes did not

exhibit a protective effect in any of six vaccinated *Aotus nancymai* monkeys (40).

A composition containing a p42 protein fused with a glutathione-S-transferase produced in *E. coli* combined with Freund's complete adjuvant did not exhibit a protective effect in two types of *Aotus* monkeys (*A. nancymai* and *A. vociferans*) when administered to them. The p19 protein produced in *S. cerevisiae* exhibited a protective effect in two *A. nancymai* type *Aotus* monkeys (12). In contrast, there was no protective effect in two *A. vociferans* type *Aotus* monkeys.

Some researchers (18) have also reported immunization tests carried out in the rabbit using a recombinant p42 protein produced in a baculovirus system and containing one amino acid sequence in common with *P. falciparum* (18). Thus these latter authors indicate that in the rabbit that recombinant p42 behaves substantially in the same way as the entire recombinant MSP-1 protein (gp195). This p42 protein in combination with Freund's complete adjuvant has been the subject matter of a vaccination test in a non-human primate susceptible to infection by *P. falciparum*, *Aotus lemurinus griseimembra* (40). The results showed that 2 of 3 animals were completely protected and the third, while exhibiting a parasitemia which resembled that of the controls, had a longer latent period. It is nevertheless risky to conclude to a protective nature in man of the antibodies thus induced against the parasites themselves. It should be remembered that there are currently no very satisfactory experimental models in the primate for *P. vivax* and *P. falciparum*. The *Saimiri* model, developed for *P. falciparum* and *P. vivax*, and the *Aotus* model for *P. falciparum*, are artificial systems requiring the parasite strains to be adapted and often requiring splenectomy of the animals to obtain significant parasitemia. As a result, the vaccination results from such models can only have a limited predictive value for man.

In any event, what the real vaccination rate would be which could possibly be obtained with such recombinant proteins is also questionable, bearing in mind the discovery - reported below - of the presence in p42s from *Plasmodiums* of the same species, and more particularly in the corresponding p33s, of hypervariable regions which would in many cases render uncertain the immunoprotective efficacy of antibodies induced in individuals vaccinated with a p42 from a *Plasmodium* strain against an infection by other strains of the same species (13).

It can even be assumed that the high polymorphism of the N-terminal portion of p42 plays a significant role in immune evasion, often observed for that type of parasite.

The aim of the present invention is to produce vaccinating recombinant proteins which can escape these difficulties, the protective effect of which is verifiable in genuinely significant experimental models or even directly in man.

More particularly, the invention provides vaccinating compositions against a *Plasmodium* type parasite which is infectious for man, containing as an active principle a recombinant protein which may or may not be glycosylated, whose essential constituent polypeptide sequence is:

- either that of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious for man, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
- or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
- or that of an immunologically equivalent peptide of said p19 fragment or said portion of that fragment; and

said recombinant protein further comprises conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognized by human antisera formed against the corresponding *Plasmodium*.

5 The presence of such conformational epitopes plays an important role in the protective efficacy of the active principle of the vaccines. They are particularly found in the active principles which exhibit the other characteristics defined above, when they are produced in a baculovirus system. If need be, it is mentioned below that the expression "baculovirus
10 vector system" means the ensemble constituted by the baculovirus type vector itself and the cell lines, in particular cells of insects transfectable by a baculovirus modified by a sequence to be transferred to these cell lines resulting in expression of that transferred sequence. Preferred examples of these two partners in the baculovirus system have been described in the
15 article by Longacre et al. (14). The same system was used in the examples below. It goes without saying, of course, that variations in the baculovirus and in the cells which can be infected by the baculovirus can be used in place of those selected.

In particular, the recombinant protein is recognized by human
20 antisera formed against the corresponding *Plasmodium* or against a homologous *Plasmodium* when it is in its non reduced state or in a reduced non irreversible state, but is not recognized or is only recognized to a slight extent by these same antisera when it is irreversibly reduced.

25 The unstable character of these conformational epitopes in a reducing medium can be demonstrated by the test described below in the examples, in particular in the presence of β -mercaptoethanol. Similarly, the examples below describe the experimental conditions applicable to obtain irreversible reduction of the proteins of the invention.

From this viewpoint, the recombinant protein produced by Longacre et al. (14) can be used in such compositions. It should be remembered that S. Longacre et al. succeeded in producing a recombinant p19 from the MSP-1 of *P. vivax* in a baculovirus vector system containing a nucleotide sequence coding for the p19 of *Plasmodium vivax*, in particular by transfecting cultures of insect cells [*Spodoptera frugiperda* (Sf9) line] with baculovirus vectors containing, under the control of the polyhedrin promoter, a sequence coding for the peptide sequences defined below, with the sequences being placed in the following order in the baculovirus vector used:

- a 35 base pair 5' terminal fragment of the polyhedrin signal sequence, in which the methionine codon for initiating expression of this protein had been mutated (to ATT);
- a 5'-terminal nucleotide fragment coding for a 32 amino acid peptide corresponding to the N-terminal portion of MSP-1, including the MSP-1 signal peptide;
- either a nucleotide sequence coding for p19, or a sequence coding for the p42 of the MSP-1 protein of *Plasmodium vivax*, depending on the case, these sequences also being provided with ("anchored" forms) or deprived of (soluble forms) 3' end regions of these nucleotide sequences, whose end C-terminal expression products are reputed to play an essential role in anchoring the final p19 protein to the parasite membrane;
- 2 TAA stop codons.

For p42, the sequences derived from the C-terminal region of MSP-1 extend consequently from amino acid Asp 1325 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) and for p19, the sequences extend from amino acid Ile 1602 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) it being

understood that the complete amino acid sequences of p42 and p19, whose initial and terminal amino acids have been indicated above follow from the gene of the Belem isolate of *P. vivax* which has been sequenced (20).

5 Similar results were obtained using, in the same vector systems, nucleotide sequences coding for the p42 and p19 of *Plasmodium cynomolgi*. The interest in *P. cynomolgi* is twofold: it is a parasitic species very close to *P. vivax* which is infectious for the macaque. It can also infect man. Furthermore, a natural host of *P. cynomolgi*, the toque macaque, is
10 accessible for testing the efficacy of the protection of MSP-1 from *P. cynomolgi* in a natural system. In addition, the rhesus monkey which is considered to be one of the most representative species for immune reactions in man, can also be infected by *P. cynomolgi*.

In particular, excellent results have been obtained in vaccination
15 tests carried out using the toque macaque with two recombinant polypeptides: soluble p42 and, in particular, soluble p19 derived from *P. cynomolgi*, respectively produced in a baculovirus system and purified on an affinity column with monoclonal antibodies recognizing the corresponding regions of the native MSP-1 protein. The following
20 observations were made: six monkeys immunized with only p19 (three monkeys) and the p19 and p42 together (three monkeys) all exhibited practically sterile immunity after challenge infection. The results obtained in the three monkeys immunized with p42 were less significant. Two of
25 them were as above, but since the third exhibited a lower parasitemia than the controls immunized with a PBS buffer in the presence of Freund's adjuvant (3 monkeys) or not immunized (3 monkeys), it was less clear.

A second challenge infection showed that the monkeys which had received p19 alone were protected for at least six months. A second vaccination test with p19 in combination with alum in this system (toque

macaque *P. cynomolgi*) exhibited significant protection for 2 of the 3 monkeys. This is the first time that MSP-1 or another recombinant antigen has demonstrated a protective effect in the presence of alum (42).

The particularly effective test results carried out with the macaque
5 with recombinant polypeptides produced in a baculovirus system using a recombinant p19 from *P. cynomolgi* showed that recombinant polypeptides respectively containing recombinant p19s from other *Plasmodiums* must behave in the same manner. They are more meaningful for malaria in man than the results from tests carried out with *P. vivax* or *P. falciparum* in their
10 "artificial hosts".

Baculovirus recombinant proteins derived from a C-terminal MSP-1 portion (p19) have a very significant antimalarial protective effect in a natural system, which constitutes the most representative model for evaluating the protective effect of MSP-1 for man.

15 The protective effect obtained can be further improved if the p19 form is deprived of the hypervariable region of the N-terminal portion of p42, the effect of which can be deleterious in natural situations in which the vaccinated subject is confronted by a great deal of polymorphism. Further, p19 appears to possess specific epitopes which are not present in p42
20 (Holm et al., 1997).

The 19 kDa C-terminal fragment, the sequence of which is present in the active principle of the vaccine, can be limited to the sequence for the p19 itself, in the absence of any polypeptide sequence normally upstream of the p19 sequence in the corresponding MSP-1 protein. Clearly, though,
25 the essential constituent polypeptide sequence of the active principle can also comprise a polypeptide sequence for the C-terminal side belonging to the 33 kDa (p33) N-terminal fragment still associated with the p19 in the corresponding p42, before natural cleavage of the latter, if the presence of this fragment does not modify the immunological properties of the active

principle of the vaccine. As will be seen below, in particular in the description of the examples, the C-terminal sequences of the p33 in various strains of the same species of *Plasmodium* (see the C-terminal portion of the peptide sequences of "region III" in Figure 4) also have a degree of
5 homology or substantial conservation of the sequence, for example on the order of at least 80%, in different varieties of *Plasmodiums* which are infectious for man, such that they do not fundamentally modify the vaccinating properties of the active principle (the sequence of which corresponds to region IV in Figure 4), in particular using the hypothesis which
10 follows from this figure; that the presumed cleavage site, between the p19 and region III of the p33 is located between the leucine and asparagine residues in a particularly well conserved region (LNVQTQ).

Normally the C-terminal polypeptide sequence of the p33, when it is present, comprises less than 50 amino acid residues, or even less than 35,
15 preferably less than 10 amino acid residues.

The essential constituent polypeptide sequence of the active principle of the vaccine need not comprise all of the sequence coding for p19, naturally providing that the latter retains the ability to induce antibodies which protect against the parasite. Preferably, this polypeptide fragment portion
20 contains the two EGF (Epidermal Growth Factor) regions.

Clearly, the skilled person could distinguish between active fragments and those which would no longer be so, in particular experimentally by producing modified vectors containing inserts with different lengths originating from the p19, respectively isolated from the fragments obtained
25 from the sequence coding for p19, by reaction with appropriate restriction enzymes, or by exonucleolytic enzymes which would be kept in contact with the fragment coding for p19 for differing periods; the capacity of the expression products from these inserts in the corresponding eukaryotic cells, in particular in insect cells, transformed by the corresponding modified

vectors, to exert a protective effect can then be tested, in particular under the experimental conditions which are described below in the examples. In particular, the expression products of these inserts must be able to inhibit a parasitemia induced *in vivo* by the corresponding whole parasite.

- 5 Thus, the invention includes all vaccinating compositions in which the essential constituent polypeptide sequence of the active principle is constituted by a peptide which can induce a cellular and/or humoral type immunological response equivalent to that produced by p19 or a fragment as defined above, provided that the addition, deletion or substitution in the
10 sequence of certain amino acids by others would not cause a large modification of the capacity of the modified peptide - hereinafter termed the "immunologically equivalent peptide" - to inhibit said parasitemia.

- The p19 fragment can naturally also be associated at the N-terminal side or the C-terminal side or via a peptide bond to a further plasmoidal
15 protein fragment having a vaccinating potential (such as Duffy binding protein from *P. vivax* (29) or EBA-175 from *P. falciparum* (30) and (31), one region of which is specifically rich in cysteine), provided that its capacity to inhibit parasitemia normally introduced *in vivo* by the corresponding parasite is not altered but is amplified.

- 20 Upstream of the N-terminal end of p19, the fragment coding for p19 or a portion thereof can also contain a peptide sequence which is different again, for example a C-terminal fragment of the signal peptide used, such as that for the MSP-1 protein. This sequence preferably comprises less than 50 amino acids, for example 10 to 40 amino acids.

- 25 These observations pertain in similar fashion to the p19 from other *Plasmodia*, in particular *P. falciparum*, the dominant species of the parasite, responsible for the most serious forms of malaria.

 However, the techniques summarized above for producing a recombinant p19 from *P. vivax* or *P. cynomolgi* in a baculovirus system are

difficult to transpose unchanged to producing a recombinant p19 of *P. falciparum* in a satisfactory yield, if only to obtain appreciable quantities which will allow immunoprotective tests to be carried out.

The invention also provides a process which overcomes this problem to a large extent. It also becomes possible to obtain much higher yields of *P. falciparum* p19 - and other *Plasmodiums* where similar difficulties are encountered - using a synthetic nucleotide sequence substituting the natural nucleotide sequence coding for the p19 of *Plasmodium falciparum* in an expression vector of a baculovirus system, this synthetic nucleotide sequence coding for the same p19, but being characterized by a higher proportion of G and C nucleotides than in the natural nucleotide sequence.

In other words, the invention follows from the discovery that expression of a nucleotide sequence coding for a p19 in a baculovirus system is apparently linked to an improved compatibility of successive codons in the nucleotide sequence to express with the "cellular machinery" of the host cells transformable by the baculovirus, in the manner of that observed for the natural nucleotide sequences normally contained in these baculovirus and expressed in the infected host cells; hence the poor expression, or even total absence of expression of a native *P. falciparum* nucleotide sequence; hence also a possible explanation of the more effective expression observed by Longacre et al. (14) for the p19 of *P. vivax* in a baculovirus system and, as the inventors have also shown, of the *P. cynomolgi* sequence from corresponding native p19 nucleotide sequences, because of their relatively much higher amounts of G and C nucleotides than those of the native nucleotide sequences coding for the p19 of *P. falciparum*.

The invention thus more generally provides a recombinant baculovirus type modified vector containing, under the control of a promoter contained in said vector and able to be recognized by cells transfectable by

said vector, a first nucleotide sequence coding for a signal peptide exploitable by a baculovirus system, characterized by a second nucleotide sequence downstream of the first, also under the control of the promoter and coding for the peptide sequence:

- 5 • either of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite other than *Plasmodium vivax* which is infectious for man, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an
10 infectious cycle;
 - or of a portion of that peptide fragment provided that the expression product from the second sequence in a baculovirus system is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
 - 15 • or of an immunologically equivalent peptide of said C-terminal peptide fragment (p19) or said peptide fragment portion by addition, deletion or substitution of amino acids not resulting in a large modification of the capacity of said immunologically equivalent peptide to induce a cellular and/or humoral type immunological response similar to that produced by
20 said p19 peptide fragment or said portion of said fragment; and
- said nucleotide sequence having, if necessary, a G and C nucleotide content in the range 40% to 60%, preferably at least 50%, of the totality of the nucleotides from which it is constituted. This sequence can be obtained by
25 constructing a synthetic gene in which the natural codons have been changed for codons which are rich in G/C without modifying their translation (maintaining the peptide sequence).

The nucleotide sequence, provided by a synthetic DNA, may have at least 10% of modified codons with respect to the natural gene sequence or

cDNA while retaining the characteristics of the natural translated sequence, i.e., maintaining the amino acid sequence.

It is not excluded that this G and C nucleotide content could be further increased provided that the modifications resulting therefrom as to the amino acid sequence of the recombinant peptide - or immunologically equivalent peptide - produced do not result in a loss of immunological properties, or protective properties, of the recombinant proteins formed, in particular in the tests which will be described below.

These observations naturally apply to other *Plasmodium* which are infectious for man, in particular those where the native nucleotide sequences coding for corresponding p19s would have T and A nucleotide contents which are poorly compatible with effective expression in a baculovirus system.

The sequence coding for the signal used can be that normally associated with the native sequence of the *Plasmodium* concerned. But it can also originate from another *Plasmodium*, for example *P. vivax* or *P. cynomolgi* or another organism if it can be recognized as a signal in a baculovirus system.

The sequence coding for p19 or a fragment thereof in the vector under consideration is, in one case, deprived of the anchoring sequence of the native protein to the parasite from which it originates, in which case the expressed protein is generally excreted into the culture medium (soluble form). It is also remarkable in this respect that under the conditions of the invention, the soluble and anchored forms of the recombinant proteins produced, in particular when they are from *P. falciparum* or *P. cynomolgi* or *P. vivax*, tend to form oligomers, this property possibly being at the origin of the increased immunogenicity of the recombinant proteins formed.

The invention also concerns vectors in which the coding sequence contains the terminal 3' end sequence coding for the hydrophobic C'-

terminal end sequence of the p19 which is normally implicated in the induction of anchoring the native protein to the cell membrane of the host in which it is expressed. This 3'-terminal end region can also be heterologous as regards the sequence coding for the soluble p19 portion, for example
5 corresponding to the 3'-terminal sequence from *P. vivax* or from another organism when it codes for a sequence which anchors the whole of the recombinant protein produced to the cell membrane of the host of the baculovirus system used. An example of such anchoring sequences is the GPI of the CD59 antigen which can be expressed in the cells of
10 *Spodoptera frugiperda* (32) type insects or the GPI of a CD14 human protein (33).

The invention also, naturally, concerns recombinant proteins, these proteins comprising conformational epitopes recognized by human serums formed against the corresponding *Plasmodium*.

15 In general, the invention also concerns any recombinant protein of the type indicated above, provided that it comprises conformational epitopes such as those produced in the baculovirus system, in particular those which are unstable in a reducing medium.

20 The invention also, naturally, concerns said recombinant proteins, whether they are in their soluble form or in the form provided with an anchoring region, in particular to cellular hosts used in the baculovirus system.

The invention also encompasses oligomers spontaneously produced in the baculovirus systems used or produced *a posteriori*, using
25 conventional protein oligomerisation techniques. The most commonly used technique involves glutaraldehyde. However, any conventional system for bridging between the respective amine and carboxyl functions in proteins can be used. As an example, any of the techniques described in European patent application EP-A-0 602 079 can be used.

The term "oligomer" means a molecule containing 2 to 50 monomer units, each of the monomer units containing p19 or a fragment thereof, as defined above, capable of forming an aggregate. The invention also encompasses any conjugation product between a p19 or a p19 fragment as defined above, and a carrier molecule - for example a polylysine-alanine -
5 for use in producing vaccines, via bonds which are covalent or otherwise. The vaccinating compositions using them also form part of the invention.

The invention still further concerns vaccine compositions using these oligomeric or conjugated recombinant proteins, including proteins from
10 *Plasmodium vivax*, these observations also extending to oligomers of these recombinant proteins.

The invention also encompasses compositions in which the recombinant proteins defined above are associated with an adjuvant, for example an alum. Recombinant proteins containing the C-terminal end region allowing them to anchor to the membrane of the cells in which they are produced are advantageously used in combination with lipids which can form liposomes appropriate to the production of vaccines. Without being limiting, lipids described, for example, in the publication entitled "Les liposomes aspects technologique, biologique et pharmacologique"
15 [Liposomes: technological, biological and pharmacological aspects] by J. Delattre et al., INSERM, 1993, can be used.

The presence of the anchoring region in the recombinant protein, whether it is a homologous or heterologous anchoring region as regards the vaccinating portion proper, encourages the production of cytophilic
25 antibodies, in particular IgG_{2a} and IgG_{2b} type in the mouse which could have a particularly high protective activity, so that associating the active principles of the vaccines so constituted with adjuvants other than the lipids used to constitute the liposome forms could be dispensed with. This amounts to a major advantage, since liposomes can be lyophilized under

conditions which enable them to be stored and transported, without the need for chains of cold storage means.

Other characteristics of the invention will become clear from the following description of examples of recombinant proteins of the invention and the conditions under which they can be produced. These examples are not intended to limit the scope of the invention.

**Description of the construction of PfMSP1_{p19}S (soluble)
(soluble p19 from *P. falciparum*)**

The recombinant construction PfMSP1_{p19}S contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32 amino acids of the MSP-1 of *Plasmodium vivax* from Met₁ to Asp₃₂ (Belem isolate; Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe due to the EcoR1 site connecting the two fragments. This is followed by the synthetic gene described in Figure 1, coding the *Plasmodium falciparum* MSP1_{p19} from Asn₁₆₁₃ to Ser₁₇₀₅ (Uganda-Palo Alto isolate; Chang et al., 1988, Exp. Parasitol., 67, 1). The construction is terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant from infected cells.

In the same manner and for comparison, a recombinant construction was produced under conditions which were similar to those used to produce the p19 above, but working with a coding sequence consisting of a direct copy of the corresponding DNA of the *P. falciparum* strain (FUP) described by Chang et al., Exp. Parasit. 67,1; 1989. The natural gene copy (from asparagine 1613 to serine 1705) was formed from the native gene by PCR.

Figure 1A shows the sequences of both the synthetic gene (Bac19) and the "native gene" (PF19).

It can be seen that 57 codons of the 93 codons of the native sequence coding for the p19 from *P. falciparum* were modified (the third nucleotide in 55 of them and the first and third nucleotides in the other 2

codons). New codons were added to the 5' end to introduce the peptide signal under the conditions indicated above and to introduce an EcoRI site for cloning, and similarly two stop codons were added which were not present in the *P. falciparum* p19 to obtain expression termination signals.

- 5 The individual letters placed above successive codons correspond to the respective successive amino acids. Asterisks (*) show the stop codons. Vertical lines indicate the nucleotides which are the same in the two sequences

**Description of the PfMSP1_{p19}A construction (anchored GPI)
(anchored p19 of *P. falciparum*)**

- 10 The PfMSP1_{p19}A construction had the characteristics of that above except that the synthetic sequence (Figure 1B) codes for the MSP1_{p19} of *Plasmodium falciparum* (Uganda-Palo Alto isolate) from Asn₁₈₁₃ to Ile₁₇₂₆ followed by two TAA stop codons. This construction gave rise to a
15 recombinant protein which was anchored in the plasma membrane of infected cells by a glycosyl phosphatidyl inositol (GPI) type structure.

Figure 1C represents the PfMSP1_{p19}S recombinant protein sequence before cutting out the signal sequence.

- 20 Figure 1D represents the PfMSP1_{p19}S recombinant protein sequence after cutting out the signal sequence.

The amino acids underlined in Figures 1C and 1D originate from the EcoRI site used to join the nucleotide sequences derived from the N-terminal portion of the MSP-1 of *P. vivax* (with signal sequence) and the MSP-1_{p19} of *P. falciparum*.

- 25 **Figure 2** - The soluble recombinant PfMSP1_{p19} antigen purified by immunoaffinity was analyzed by immunoblot using SDS-PAGE in the presence (reduced) or absence (non reduced) of β -mercaptoethanol. Samples were charged onto gel after heating to 95°C in the presence of 2% SDS. Under these conditions only covalent type bonds (disulphide

bridges) can resist disaggregation. The left hand blot was revealed with a monoclonal antibody which reacted with a linear epitope of natural p19. The right hand blot was revealed with a mixture of 13 human antisera originating from subjects with acquired immunity to malaria due to *Plasmodium falciparum*. These results show that the recombinant baculovirus molecule can reproduce conformational epitopes in the form of a polymer the majority of which are recognized by human antiserum.

Figure 2B: Immunoblot analysis with human antiserum of recombinant purified MSP-1 p19 from *P. vivax* and *P. cynomolgi* under non reduced (NR), reduced only in the charging medium (R) and irreversibly reduced (IR) conditions:

This work was based on the idea that the baculovirus expression system correctly reproduced the conformational epitopes present *in vivo* on the C-terminal portion of MSP-1 in large amounts. The best means of measuring this property (which may be the only possible means in the absence of native purified proteins corresponding to p19) was to study the reactivity of the recombinant proteins with the antiserum of individuals exposed to malaria, this reflecting the native proteins as "seen" by the human immune system.

Thus soluble recombinant PvMSP-1 p19 and PcMSP-1 p19 antigens purified by immunoaffinity were analyzed by immunoblot using SDS-PAGE (15%) in the presence (reduced) or absence (non reduced) of DTT. Samples were loaded onto the gel after heating to 95°C in the presence of 2% SDS. The irreversible reduction was carried out as follows: the protein was resuspended in 0.2 M Tris-HCl, pH 8.4, 100 mM DTT, 1.0% SDS and heated for 30 minutes at 70°C. After diluting with water, acrylamide was added to a final concentration of 2 M and the mixture was incubated under nitrogen in the dark for 1 hour at 37°C. The immunoblot was revealed with a mixture of 25 human antisera originating from subjects with an acquired

immunity to malaria due to *Plasmodium vivax*. V and C respectively designate proteins derived from the MSP-1 of *P. vivax* and *P. cynomolgi*. It should be noted that irreversibly reduced recombinant proteins exhibited no reactivity with human antiserum while non irreversibly reduced proteins or
5 non reduced proteins exhibited good reactivity. (The non reduced Pv MSP-1 p19 was a little weak since in its glycosylated state it does not bind well to nitro-cellulose paper). These results show that recognition of baculovirus MSP-1 p19 molecules by human antiserum is largely if not completely dependent on conformational epitopes sensitive to reduction
10 which are reproduced in this system.

Figure 3 - The soluble PvMSP1_{p42} recombinant antigen (Longacre et al., 1994, op. Cit.) was incubated for 5 hours at 37°C in the presence of protein fractions derived from merozoites of *P. falciparum* and separated by isoelectrofocussing. The samples were then analyzed by immunoblot in the
15 presence (reduced) or absence (non reduced) of β -mercaptoethanol. Isoelectrofocussing fractions 5 to 12, and two total merozoite extracts made in the presence (Tex) or absence (T) of detergent, were analyzed. The immunoblot was revealed with monoclonal antibodies specific for MSP1_{p42} and p19 of *P. vivax*. The results suggest that there is a proteolytic activity in
20 the *P. falciparum* merozoites which can be extracted with detergent. Digestion of p42 in certain fractions appear to cause polymerization of the digestion products (p19); this polymerization is probably linked to the formation of disulphide bridges since in the presence of β -mercaptoethanol, the high molecular weight forms disappear in favor of a molecule of about
25 19 kDa (Tex-R). The p19 polymerization observed in these experiments could thus be an intrinsic property of this molecule *in vivo*.

Figure 3B: The differential contribution of p42 and p19 antigens to the *P. vivax* anti-MSP-1 human response.

Recognition of *P. vivax* MSP-1 p42 and p19 antigens by the antiserum of individuals with an acquired immunity to *P. vivax* was compared using the ELISA inhibition technique as follows: a mixture of 25 human antisera originating from subjects with an acquired immunity to malaria due to *P. vivax* was diluted to 1:5000 and incubated for 4 hours at ambient temperature either alone, or in the presence of a 1 mM purified *P. vivax* recombinant p42 or p19. This mixture was transferred to a microtiter well which had been coated for 18 hours at 4°C with 500 ng.ml⁻¹ of purified absorbed recombinant p42 or p19, and incubated for 30 minutes at ambient temperature. After washing with PBS containing 0.1% of Tween 20, a goat anti-mouse IgG conjugated with peroxidase was added and the mixture was incubated for 1 hour at 37°C. The enzymatic activity was revealed by reading the optical density at 492 nm. The percentage inhibition was calculated based on values of 100% of antiserum activity with the coated antiserum on the microtiter plate in the absence of a competing antigen. Statistical data were calculated using a Statview program. Each bar represents the average percentage inhibition of a pair of competing/absorbed antigens based on 4 to 12 determinations; the vertical lines correspond to a 95% confidence interval. Asterisks (*) designate the antigens produced in the presence of tunicamycin, thus with no N-glycosylation. The important parameters of these measurements were the dilution of the antiserum by 1:5000 which is in the region which is sensitive to ELISA curves and the competing antigen concentrations of 1 mM which includes competition by low affinity epitopes. Thus these data reflect the maximum resemblance between the two compared antigens. The results show that the majority, if not all of the p42 epitopes recognized by the human antiserum are present on the p19 since in the presence of the latter, the reactivity of the human antiserum against p42 is inhibited as much as by the p42 antigen itself. In contrast, however, about 20% of the p19

epitopes recognized by human antiserum were not or were not accessible on the p42, since the reactivity of the human antiserum against the p19 was much less inhibited by p42 than by p19 itself. Such specific epitopes of p19 could be constituted or revealed only after cleaving the p42 into p19 and p33. These results were not affected by glycosylation showing that the effect is really due to a difference between the peptide components of p19 and p42 and not to a difference in glycosylation. These results underline the fact that p19 has a distinct immunological identity compared to p42.

Description of the PcMSP1_{p19}S (soluble) construction (soluble p19 of *P. cynomolgi*)

The DNA used for the above construction was obtained from a clone of the *Plasmodium cynomolgi* ceylonensis strain (22-23). This strain had been maintained by successive passages through its natural host (*Macaca sinica*) and cyclic transmissions via mosquitoes (27).

Blood parasites in the mature schizont stage were obtained from infected monkeys when the parasitemia had attained a level of 5%. They were then purified using the methods described in (25). The DNA was then extracted as described in (26).

A 1200 base pair fragment was produced using a PCR reaction using the oligonucleotides underlined in Figure 4 originating from *P. vivax*. The 5' oligonucleotide comprised an EcoRI restriction site and the 3' oligonucleotide comprised two synthetic TAA stop codons followed by a BglII restriction site. This fragment was introduced by ligation and via these EcoRI and BglII sites into the pVLSV₂₀₀ plasmid already containing the signal sequence for the MSP-1 protein of *P. vivax* (19). The new plasmid (pVLSV₂₀₀C₄₂) was used to analyze the DNA sequences.

The *P. cynomolgi* and the corresponding *P. vivax* sequences were aligned. The black arrows designate the presumed primary and secondary cleavage sites. They were determined by analogy with known sites in *P.*

falciparum (27, 28). The vertical lines and horizontal arrows localize the limits of the four regions which were studied. Region 4 corresponded to the sequence coding for the *P. cynomolgi* p19. Glycosylation sites are boxed and the preserved cysteines are underlined. The lower portion of Figure 4 shows the percentage identity between the two isolates of *P. vivax* and *P. cynomolgi*.

The recombinant construction PcMSP1_{p19}S contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32 amino acids of the MSP-1 of *Plasmodium vivax* from Met₁ to Asp₃₂ (Belem isolate; Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe, due to the EcoR1 site, connecting the two fragments. This is followed by the sequence coding for the *Plasmodium cynomolgi* MSP1_{p19} from Lys₂₇₆ to Ser₃₈₀ (Ceylon strain). The construction was terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant of infected cells.

Purification of recombinant PfMSP1p19 protein by immunoaffinity chromatography with a monoclonal antibody specifically recognizing the p19 of *Plasmodium falciparum*

The chromatographic resin was prepared by binding 70 mg of a monoclonal antibody (obtained from a G17.12 hybridoma deposited at the CNCM [National Collection of Microorganism Cultures] (Paris, France) on the 14th February 1997, registration number I-1846; this G17.12 hybridoma was constructed from X63 Ag8 653 myeloma producing IgG 2a/k recognizing the *P. falciparum* p19) to 3 g of activated CNBr-Sepharose 4B (Pharmacia) using standard methods detailed in the procedure employed by Pharmacia. The culture supernatants containing the soluble PfMSP1p19 were batch incubated with the chromatographic resin for 16 hours at 4°C. The column was washed once with 20 volumes of 0.05% NP40, 0.5 M of NaCl, PBS; once with 5 volumes of PBS and once with 2 volumes of 10 mM of sodium

phosphate, pH 6.8. Elution was carried out with 30 ml of 0.2 M glycine, pH 2.2. The eluate was neutralized with 1 M sodium phosphate, pH 7.7 then concentrated by ultrafiltration and dialyzed against PBS. To purify the anchored PfMSP1p19, all of the washing and elution solutions contained a supplemental 0.1% of 3-(dimethyl-dodecylammonio)-propane sulphonate (Fluka).

**Recombinant *Plasmodium vivax* (p42 and p19) MSP1 vaccination test
in the squirrel monkey *Saimiri sciureus***

This vaccination test was carried out on male non splenectomized 2 to 3 year old *Saimiri sciureus boliviensis* monkeys. Three monkeys were injected 3 times intramuscularly at 3 week intervals with a mixture of about 50 to 100 µg each of recombinant soluble PvMSP1_{p42} and p19 (19), purified by immunoaffinity. Complete and incomplete Freund's adjuvant was used as follows: 1st Injection: 1:1 FCA/FIA; 2nd injection: 1:4 FCA/FIA; 3rd injection: FIA. These adjuvant compositions were then mixed 1:1 with the antigen in PBS. Five control monkeys received the glutathione-S-transferase (GST) antigen produced in *E. coli* using the same protocol. The challenge infection was carried out by injecting 2×10^6 red blood cells infected with an adapted *Plasmodium vivax* strain (Belem) 2.5 weeks after the final injection. The protection was evaluated by determining parasitemia daily in all animals by examining smears stained with Giemsa.

The curves in Figure 5 show the variation in the measured parasitemia as the number of parasitic red blood cells per microliter of blood (logarithmic scale on the ordinate) as a function of the time passed after infection (in days). Curve A corresponds to the average values observed in the three vaccinated monkeys; curve B corresponds to the average values in the five control monkeys.

An examination of the Figure shows that the effect of the vaccination was to greatly reduce the parasitism.

**Recombinant *Plasmodium cynomolgi* (p42 and p19) MSP1 vaccination
test in the toque macaque *Macaca sinica***

Fifteen captured monkeys were used as follows: (1) 3 animals injected with 100 µg of soluble PcMSP1_{p42}; (2) 3 animals injected with 35 µg (1st injection) or 50 µg (2nd and 3rd injections) of soluble PcMSP1_{p42}; (3) 3 animals injected with a mixture of PcMSP1_{p42} and p19; (4) 3 animals injected with adjuvant plus PBS; (5) 3 animals not injected. Complete and incomplete Freund's adjuvant was used in the protocol described above. Injections were intramuscular at 4 week intervals. The challenge infection was made by injecting 2×10^5 red blood cells infected with *Plasmodium cynomolgi* 4 weeks after the last injection. Protection was evaluated by determining parasitemia daily in all animals by examining the parasitemia with Giemsa. Parasitemia were classified as negative only after counting 400 smear fields. The parasitemia were expressed as a percentage of parasitised red blood cells.

Figures 6A - 6G show the results obtained. Each of them shows parasitemia (expressed as the percentage of parasitised red blood cells along the ordinate on a logarithmic scale) observed in the challenge animals as a function of the time after infection (in days along the abscissa).

The results relate to:

- in Figure 6A; non vaccinated control animals;
- Figure 6B relates to animals which received a saline solution also containing Freund's adjuvant;
- Figure 6C is a superposition of figures 6A and 6B, with the aim of highlighting the relative results resulting from administration of Freund's adjuvant to the animals (the variations are clearly not significant);
- Figure 6D provides the results obtained after vaccination with p42;
- Figure 6E concerns animals vaccinated with p19 alone;

- finally, **Figure 6F** concerns animals vaccinated with a mixture of p19 and p42.

The p42 certainly induced a certain level of protection. However, as shown in Figures 6E and 6F, the protection conferred by the recombinant p19 of the invention was considerably better.

The numbers used to produce graphs (6A-6F) are given in **Figure 6G**.

***P. cynomolgi* toque macaque vaccination test; second challenge
infection of monkeys vaccinated with p19 alone
and controls (Figures 8)**

Six months later, with no other vaccination, the 3 macaques which received the p19 MSP-1 alone with FCA/FIA (Figure 6E) and the 3 macaques which received a saline solution containing Freund's adjuvant (Figure 6B) and 2 new unvaccinated monkeys underwent a new challenge infection by injecting 1×10^6 red blood cells infected with *Plasmodium cynomolgi*. Protection was evaluated by determining parasitemia daily in all animals by examining Giemsa smears. The parasitemia were classified negative only after counting 400 smear fields. The parasitemia were expressed as the percentage of parasitised red blood cells (the figures used to produce graphs 8A-C are given in **Figure 8D**). The six immunized animals which underwent challenge infection six months earlier had no detectable parasitemia except for 1 animal in each group which exhibited a parasitemia of 0.008% for 1 day (**Figures 8A and 8B**). The two unaffected controls exhibited a conventional parasitemia with a maximum of 0.8% and for 21 days (**Figure 8C**). Thus the 3 animals vaccinated with the MSP-1 p19 were as well protected six months later as the 3 controls which exhibited a complete conventional infection after the first challenge infection, despite the absence of or a very slight parasitemia after the first challenge infection. (It is likely that protection against a homologous strain

of *P. vivax* in humans can also be induced by a single blood infection provided that the infection is allowed to run its natural course without treatment since untreated *P. vivax* infection in humans gave rise to complete clinical protection against the subsequent challenge with the identical strain but not with a different strain of the same species).

Together these results suggest either that the protection period for p19 is at least six months or that the immunity induced can be effectively boosted by minimal exposure to parasitic infection.

Vaccination test with p19 in association with alum in the *P. cynomolgi* toque macaque system (Figures 9)

The previous positive protection results were obtained using complete (FCA) or incomplete (FIA) Freund's adjuvant. However, the only adjuvant which is currently allowed in man is alum. For this reason, we carried out a vaccination test with *P. cynomolgi* MSP-1 p19 in the toque macaque in the presence of alum as the adjuvant. Six captured macaques were used as follows: (1) 3 animals injected with 4 doses of 50 mg of recombinant *P. cynomolgi* MSP-1 p19 with 10 mg of alum; (2) 3 animals injected 4 times with physiological water and 10 mg of alum. The injections were intramuscular at 4 week intervals. The challenge infection was made by injecting 2×10^5 red blood cells infected with *P. cynomolgi* 4 weeks after the last injection. Protection was evaluated by daily determination of parasitemia in all animals by examining Giemsa smears. The parasitemia were classified negative only after counting 400 smear fields. Parasitemia were expressed as the percentage of parasitised red blood cells. The results of this experiment were as follows. 2 of the 3 macaques immunized with recombinant p19 with alum had about 30 times less total parasitemia during the infection period (Figures 9A and 9B) than the 3 control macaques immunized with physiological water and alum (Figure 9D) after the challenge infection. The third macaque immunized with p19 (Figure

9C) was not very different from the controls. For the vaccination test using *Plasmodium cynomolgi* p19 in the toque macaque, *macaca sinica*, described in Figure 9, the data used to produce the graphs (9A-9D) are given in (Figure 9E). While the results are a little less spectacular than the preceding results (Figures 6, 8), this is the first time that significant protection has been observed for recombinant MSP-1 with alum.

Figure 10: Vaccination test with a recombinant *Plasmodium falciparum* p19 in the squirrel monkey

Twenty *Saimiri sciureus guyanensis* (squirrel monkeys) of about 3 years old raised in captivity were used as follows: (1) 4 animals injected with 50 µg of soluble Pf MSP-1 p19 in the presence of Freund's adjuvant as follows: 1st injection: 1:1 FCA/FIA; 2nd injection: 1:4 FCA/FIA; 3rd injection: FIA. These adjuvant compositions were then mixed with 1:1 antigen in PBS; (2) 2 control animals received Freund's adjuvant as described for (1) with only PBS; (3) 4 animals injected with 50 µg of soluble Pf MSP-1 p19 in the presence of 10 mg of alum (Alu-Gel-S, Serva); (4) 2 control animals received 10 mg of alum with only PBS; (5) 4 animals injected with about 50-100 mg of GPI anchored Pf MSP-1 p19 reconstituted into liposomes as follows: 300 mmoles of cholesterol and 300 mmoles of phosphatidyl choline were vacuum dried and resuspended in 330 mM of N-octylglucoside in PBS with 1.4 mg of Pf MSP-1 p19, GPI. This solution had been dialyzed against PBS with adsorbent Bio-Beads SM-2 (Bio-Rad) and the liposomes formed were concentrated by centrifuging and resuspended in PBS. The 1st injection was made with fresh liposomes kept at 4°C and the 2nd and 3rd injections were made with liposomes which had been frozen for preservation; (6) 2 animals injected with control liposomes made in the same way, in the absence of the p19, GPI antigen as described for (5); (7) 2 animals injected with physiological water. Three intramuscular injections were made at 4 week intervals. The challenge infection was made by

injecting 1×10^6 red blood cells infected with *Plasmodium falciparum*. Protection was evaluated by determining parasitemia daily in all animals by examining the Giemsa smears. Parasitemia were expressed as the percentage of parasitised red blood cells. The results of this vaccination test are shown in Figures 10, A-G.

The groups immunized with p19 in Freund's adjuvant or liposome demonstrated similar parasitemia to the control groups after a challenge infection (one animal (number 29) vaccinated with p19 in Freund's adjuvant died several days after challenge infection for reasons independent of vaccination (cardiac arrest). Irregularities in administration of the antigen in these 2 groups (poor Freund's emulsion, congealed liposomes) did not allow the significance of these results to be completely evaluated. In the alum group, 2 animals showed total parasitemia for the duration of the infection about 8 times less than the controls, 1 animal about 3 times less and 1 animal was similar to the controls. This experiment was a little difficult to interpret due to the variability in the controls, probably due to the strain of parasite used for the challenge infection which would not have been quite adapted to the non splenectomized *Saimiri* model developed only recently in Cayenne. However, the real effect with alum, although imperfect, is encouraging in that our antigens seem to be the only recombinant *P. falciparum* MSP-1 versions which currently have shown a certain effectiveness in combination with alum.

**Figure 7: Specific reactivity with *P. falciparum* MSP1 p19 monkey
anti-sera with high molecular weight aggregates
(same antisera as for Figures 10)**

Monkeys bred in captivity were injected intramuscularly with 1 ml of
5 inoculum twice at 4 week intervals as follows: (1) 4 animals injected with
50 µg of soluble PfMSP1p19 in the presence of Freund's adjuvant as
follows: 1st injection: 1:1 FCA/FIA; 2nd injection: 1:4 FCA/FIA; and mixed
then 1:1 with the antigen in PBS; (2) 4 animals injected with 50 µg of
soluble PfMSPp19 in the presence of 10 mg of alum; (3) 4 animals injected
10 with about 50 µg of GPI anchored PfMSP1p19 reconstituted into liposomes
composed of 1:1 molar cholesterol and phosphatidyl choline. The animals
were bled 17 days after the second injection.

Red cells from a squirrel monkey with 30% parasitemia due to *P.*
falciparum (with the mature forms in the majority) were washed with PBS
15 and the residue was diluted 8 times in the presence of 2% SDS and 2%
dithiothreitol and heated to 95° before being charged onto a polyacrylamide
gel of 7.5% (separation gel) and 4% (stacking gel). After transfer to
nitrocellulose, immunoblot analysis was carried out with antisera as follows:
(1) pool of antisera of 4 monkeys vaccinated with soluble PfMSP1p19 in
20 Freund's adjuvant, 1:20 dilution; (2) pool of antisera of 4 monkeys
vaccinated with soluble PfMSP1p19 in alum adjuvant, 1:20 dilution; (3) pool
of antisera of 4 monkeys vaccinated with anchored PfMSP1p19 in
liposomes, 1:20 dilution; (4) monoclonal antibody, which reacts with a linear
epitope of PfMSP1p19, 50 µg/ml; (5) SHI90 antisera pool originating from
25 about twenty monkeys repeatedly infected with *P. falciparum* and which
had become resistant to any subsequent infection with *P. falciparum*, 1:500
dilution; (6) antiserum pool of unaffected monkeys (never exposed to *P.*
falciparum), 1:20 dilution.

The results in Figure 7 show that the 3 antisera pools of monkeys vaccinated with PfMSP1p19 either with Freund's adjuvant (track 1), alum (track 2) or in liposomes (track 3) reacted specifically with very high molecular weight complexes (diffuse in the stacking gel) and present in parasite extracts containing more mature forms. These results support the hypothesis that a specific aggregate of PfMSP1p19 is present *in vivo* comprising epitopes which are reproduced in recombinant PfMSP1p19 molecules synthesized in the baculovirus system, in particular oligomeric forms thereof.

In Figure 7B: The techniques and methods were the same as for Figure 7 except that the individual antiserum for each monkey was tested after three injections the day of the challenge infection and the SHI antiserum was diluted by 1:250. The numbers correspond to the individual monkeys noted in Figure 10. The results show that the antiserum for 4 monkeys vaccinated with p19 and alum reacted strongly and specifically with very high molecular weight complexes while the monkeys of other groups vaccinated with p19 and Freund's adjuvant or liposomes showed only a little reactivity as in the control reactivity with these complexes. Since the monkeys vaccinated with p19 and alum were also the best protected, this reactivity with the high molecular weight complexes appeared to indicate a protective effect, despite one monkey in the group not being protected with respect to the controls and that another was only partially protected.

The invention also, of course, concerns other applications, for example those described below with respect to certain of the examples, although these are not limiting in character.

Therapy

The recombinant molecule PfMSP1p19 can be used to produce specific antibodies which can possibly be used by passive transfer for therapeutics for severe malaria due to *P. falciparum* when there is a risk of death.

Diagnostics

The recombinant molecules PvMSP1p42, PvMSP1p19 and PfMSPp19 derived from baculovirus can and have been used to produce specific murine monoclonal antibodies. These antibodies, in combination with polyclonal anti-MSP1p19 antisera originating from another species such as the rabbit or goat can form the basis of a semi-quantitative diagnostic test for malaria which can distinguish between malaria due to *P. falciparum*, which can be fatal, and malaria due to *P. vivax*, which is generally not fatal. The principle of this test is to trap and quantify any MSP-1 molecule containing the p19 portion in the blood.

In this context, the advantages of the MSP1p19 molecule are as follows:

- (i) it is both extremely well conserved in the same species and sufficiently divergent between different species to enable specific species reactants to be produced. No cross reaction has been observed between antibodies derived from PfMSP1p19 and PvMSP1p19;
- (ii) the function of MSP1p19, while not known with precision, seems to be sufficiently important that this molecule does not vary significantly or is deleted without lethal effect for the parasite;
- (iii) It is a major antigen found in all merozoites and thus it must in principle be detectable even at low parasitemia and proportionally to the parasitemia;
- (iv) since the recombinant MSP1p19 molecules derived from baculovirus appear to reproduce more of the native structure of MSP1p19, the

antibodies produced against these proteins will be well adapted to diagnostic use.

The microorganisms identified below have been deposited at the Collection Nationale de Culture de Microorganismes de L'Institut Pasteur (CNCM) under Rule 6.1 of the Treaty of Budapest on 1st February 1996, under the following registration numbers:

<u>Identification reference</u>	<u>Registration numbers</u>
PvMSP1p19A	I-1859
PvMSP1p19S	I-1860
PfMSP1p19A	I-1861
PfMSP1p19S	I-1862
PcMSP1p19S	I-1863

The microorganism identified below has been deposited at the CNCM under Rule 6.1 of the Treaty of Budapest on July 18, 1998 under the following registration number:

<u>Identification reference</u>	<u>Registration number</u>
P1MSP1p19S; His Ext; Sig Pf	I-2041

The invention also concerns the use of these antibodies, preferably fixed to a solid support (for example for affinity chromatography) for the purification of type p19 peptides initially contained in a mixture.

Purification means bringing this mixture into contact with an antibody, dissociating the antigen-antibody complex and recovering the purified p19 type peptide.

The invention also concerns vaccine compositions, also comprising mixtures of proteins or fragment, in particular mixtures of the type:

- *P. falciparum* p19 and *P. vivax* p19;
- *P. falciparum* p19 and *P. falciparum* p42, the latter if necessary being deprived of its most hypervariable regions;
- *P. vivax* p19 and *P. vivax* p42, the latter if necessary being deprived of its most hypervariable regions;
- *P. falciparum* p19 and *P. falciparum* p42, the latter if necessary being deprived of its most hypervariable regions, and *P. vivax* p19 and *P. vivax* p42, the latter if necessary being deprived of its most hypervariable regions.

In the present case, the most hypervariable regions are defined as region II or region II and all or part of region III, the portion of region III which is preferably deleted being that which is juxtaposed to region II (the conserved portion being located to the side of the C-terminal of p33, close to the p19). Regions II and III are illustrated in Figure 4.

The invention is not limited to the production of human vaccine. It is also applicable to the production of veterinary vaccine compositions using the corresponding proteins or antigens derived from parasites which are infectious for mammals and products under the same conditions. It is known that infections of the same type, babesiosis, also appear in cattle, dogs and horses. One of the antigens of the *Babesia* species has a high conformational homology (in particular in the two EFG-like and cysteine-rich domains) and functional homology with a protein portion of MSP-1 [(36), (37) and (38)].

Examples of veterinary vaccines using a soluble antigen against such parasites have been described (39).

It goes without saying that the p19s used in these mixtures can also be modified as described in the foregoing when considered in isolation.

The invention also concerns hybridomas secreting specific antibodies selectively recognizing the p19 of a MSP-1 protein in the merozoite form of a *Plasmodium* type parasite which is infectious for man other than *Plasmodium vivax* and which does not recognize *Plasmodium vivax*.

In particular, these hybridomas secrete monoclonal antibodies which do not recognize *Plasmodium vivax* and which specifically recognize *Plasmodium falciparum* p19.

The invention also concerns a hybridoma characterized in that it produces a specific antibody which specifically recognizes the p19 of *P. vivax* and the p19 of *P. cynomolgi*. A F10-3 hybridoma has been constructed from the X63 Ag8 653 myeloma producing IgG 2b/k recognizing the p42 glycoprotein of *Plasmodium vivax*. The F10-3 hybridoma has been deposited at the European Collection of Cell Cultures (ECACC) under Rule 6.1 of the Treaty of Budapest on August 6, 1998 under the Accession Number: 98080510.

It should be noted that in the text which follows as well as in the Figures whose numerals are headed by numbers 11, 12, 13, 14 and 15 respectively the references to MSP₁₁₉ and MSP₁₄₂ stand for p19 and p42 as they have been defined hereabove.

Of particular significance is the high degree of purity of the recombinant p19 proteins as obtainable by expression in baculovirus systems and Immunoaffinity or metallo-affinity chromatography.

Particularly, the recombinant p19 protein is crystallizable implicating an absolute purity. In addition, mass spectrometry measurements also show a very high degree of purity estimated at greater than 95%.

Of particular significance also are the reduction-sensitive conformational epitopes in the two EGF domains of the preferred p19 recombinant proteins and the long term memory response directed in a

substantially specific manner against said conformational epitopes, which said p19 recombinant proteins are capable of eliciting in laboratory animals.

These properties as well as other characteristics of preferred purified p19 recombinant proteins are further illustrated by the results provided by further studies which are reported hereafter.

The invention relates also particularly to recombinant proteins, as obtainable in a baculovirus vector system:

- In a pure state
- substantially free of any other form of recombinant protein which, has the same peptide sequences, but which contains alternate conformations in the two EGF regions. This alternate conformation is different from the conformational form as defined by:
 - (a) the atomic coordinates as detailed in Annexes I, II or II obtained by crystallography (the Annexes I, II and II include respectively the atomic coordinates which define the *P. cynomolgi* MSP119, *P. vivax* MSP119, and *P. falciparum* MSP119 three-dimensional molecular structure); and
 - (b) the NMR fingerprints as illustrated in Figures 12.0a to 12.2c.

The following data emphasize the importance of reduction-sensitive conformational epitopes in the two EGF domains comprising the MSP119 antigens. The quantitative reproduction of a single conformation likely to resemble the native parasitic protein in *Plasmodium* C-terminal MSP119 recombinant proteins derived from a higher order expression system such as baculovirus (indicated by crystal formation as described below), is thought to be one of the essential active principles necessary for the protective effect of these antigens in the vaccination trials described previously. Three types of experimental data are included:

(i) The spatial organization of the baculovirus recombinant MSP119 molecule from *Plasmodium cynomolgi* as defined by the determination of its crystal structure at 1.8 Å resolution. The probable structures of the corresponding MSP119 antigens derived from *Plasmodium vivax* and *Plasmodium falciparum* were determined by molecular modeling by the replacement of alternative residues present in the latter two species as compared to the *P. cynomolgi* MSP119.

(ii) The nuclear magnetic resonance (NMR) spectra for each of the 3 MSP119 recombinant proteins derived from *P. cynomolgi*, *P. vivax* and *P. falciparum*. These spectra represent precise, defined "fingerprints" of the conformation of the corresponding proteins in solution.

(iii) ELISA data indicating that the immune response to the recombinant MSP119 molecules is almost entirely directed against reduction-sensitive conformational epitopes both in humans exposed to the malaria parasite and in the highly protected toque monkeys inoculated with the *P. cynomolgi* p19 recombinant protein (Figure 8E). These results underline the importance of accurate and quantitative reproduction of these epitopes in the recombinant proteins designed to elicit a protective anti-malaria immune response. In addition it is shown that vaccination with the MSP142 recombinant protein favors a long term memory response which is preferentially directed against non-conformational epitopes present in the MSP142 in contrast to the MSP119 whose long term memory response is also directed against the conformational epitopes associated with protection.

Reference is made hereafter to the sets of drawings headed by numerals 11, 12, 13, 14 and 15, and to which the following legends respectively correspond.

Figure 11A: Backbone of MSP119 from *P. cynomolgi* showing disulfide bridges in bold line.

Figure 11B: Backbone of MSP119 showing positions of sequence differences between *P. cynomolgi* and *P. vivax*.

5 **Figure 11C:** Backbone of homology-modeled MSP119 of *P. falciparum* with positions of sequence differences with *P. cynomolgi*.

Figure 12: Reconstructed mass spectra and m/z spectra respectively of metalloaffinity purified *P. cynomolgi* (A,B), *P. falciparum* (C,D) and *P. vivax* (E,F) MSP119.

10 **Figure 12.0a to 12.0c:** Regions of the NOESY (a and b) and TOCSY (c) spectra of *P. cynomolgi* MSP119. The chemical shifts in the F1 and F2 dimensions are expressed in parts per million (ppm). (a) and (b): crosspeaks outside the diagonal correspond to dipolar interactions between two hydrogen nuclei of the protein. Signals at 4.67 ppm in (a) correspond to the residual water signal, and probably to dipolar interactions of H nuclei of the protein resonating at the same frequency as the water protons. (c): crosspeaks correspond to through-bond interactions between hydrogen nuclei in an amino acid residue. As in the NOESY spectrum, the peaks at 4.67 ppm arise from the residual water signal and probably from
15 interactions of two nuclei in the same residue. Twenty positive contours are plotted at a vertical scale of 5000 (a, b) or 15000 (c) and a threshold level of 7 (a, b) or 6 (c) using the VNMR 5.3 software.

Figure 12.1: Regions of the NOESY (a and b) and TOCSY (c) spectra of *P. vivax* MSP119. Twenty positive contours are plotted at a vertical scale of
20 5000 (a), 4000 (b) or 30000 (c) and a threshold level of 7 (a, b) or 6 (c) using the VNMR 5.3 software.

Figure 12.2: Regions of the NOESY (a and b) and TOCSY (c) spectra of *P. falciparum* MSP119. Twenty positive contours are plotted at a vertical scale

of 5000 (a), 4000 (b) or 10000 (c) and a threshold level of 7 (a, b) or 6 (c) using the VNMR 5.3 software.

Figure 13: ELISA titration of tertiary monkey anti *P. cynomolgi* recombinant MSP1 antisera. Plates were coated either with native (N) or reduced, denatured (D) *P. cynomolgi* MSP119 or MSP142. OD; Optical density at 492 nm. Figures A to F represent respectively, monkeys 426-427-429 (anti- MSP119) and 428-434-435 (anti- MSP142) (Perera et al. 1998).

Figure 14: ELISA analysis of human *P. vivax* infected donors under reducing and non reducing conditions using immunoaffinity (A) or metalloaffinity purified (B) MSP119 coating antigen.

Figure 15: ELISA titration of murine anti *P. cynomolgi* MSP119 and MSP142 antisera.

(i) Crystallization and structure determination

The expression of recombinant MSP119 in baculovirus is described by Holm et al., 1997 which is hereby incorporated by reference. Modified versions of the constructs described were produced by including a carboxyterminal hexahistidine tag to facilitate purification by metalloaffinity chromatography. Culture supernatants from spinner cultures were harvested and dialyzed extensively against 5 mM Tris-HCl, pH 8.0 at 4°C. The dialysate was adjusted to 20 mM Tris, pH 8.0, 0.1 M NaCl (loading buffer) and passed on columns of "Talon" metalloaffinity resin (Clontech, Palo Alto, U.S.A.) previously equilibrated with loading buffer. The charged resin was washed with loading buffer and eluted with the same buffer supplemented with 100 mM imidazole, pH 8.0. Eluted fractions were pooled and dialyzed against 10 mM potassium phosphate, pH 7.2.

Crystals were grown from a buffer containing 30% PEG 4000 (wt./vol.), 0.2 M ammonium acetate and 0.1 M sodium citrate at pH 5.6. The crystals belong to the trigonal space group P3₂2₁ (the enantiomorph

P3121 was excluded during the structure analysis; see below) with unit cell dimensions $a = b = 43.77 \text{ \AA}$, $c = 92.04 \text{ \AA}$ and with one molecule of MSP119 accommodated in the asymmetric unit.

All X-ray data were recorded with a MAR-Research image plate at the synchrotron beam line DW32, LURE, Orsay. Native diffraction data were obtained at 18°C from one crystal with dimensions $0.4 \times 0.2 \times 0.2 \text{ mm}$ using an X-ray wavelength of 0.970 \AA . A total of 139,331 Bragg intensities were measured between 15.0 to 1.8 \AA resolution, which reduced to 9980 unique reflections upon merging equivalent reflections, corresponding to a complete data set within this resolution range. A single heavy-atom derivative was prepared by soaking a crystal overnight in a solution of p-chloromercurypheyl sulphonic acid dissolved at a concentration of 6 mM in the crystallization buffer. Diffraction data were recorded at 18°C from one crystal, similar in size to that used as native, with an X-ray wavelength of 0.997 \AA . The 57,824 individual intensity measurements reduced to a unique data set of 3905 reflections that was complete between the resolution limits of 20.0 and 2.5 \AA . The unit cell dimensions of the derivative remained close to those of the native: $a = b = 43.93 \text{ \AA}$, $c = 92.57 \text{ \AA}$.

Isomorphous and anomalous difference Pattersons both gave peaks consistent with a single heavy-atom site. The mercury parameters were refined with the program SHARP (La Fortelle and Bricogne, 1997) using isomorphous and anomalous structure amplitude differences between the resolution limits of 13.0 and 2.5 \AA . The figure of merit and phasing power was 0.56 and 1.7 , respectively, for the acentric data, and 0.41 and 1.4 , respectively, for the centric data. The correct space group enantiomorph was distinguished during phase refinement by solvent flattening with the program SOLOMON (Abrahams and Leslie, 1996); the final overall R-factor was 0.316 for P3121 and 0.223 for P3221, thus clearly indicating the latter space group.

The polypeptide chain could be readily traced for the whole of the first domain. By contrast, the second domain presented certain difficulties owing to lack of continuity of the electron density for some regions of the polypeptide chain. An electron density map offering a more facile interpretation of the second domain was subsequently obtained by combining the probability distributions of calculated phases from the traced structure of the first domain with those of SIRAS phases (using the program SIGMMA (Read, 1986)), followed by density modification of the resulting Fourier synthesis (using the program DM (Cowtan, 1994; Bayley, The CCP4 suite, 1994)). The atomic parameters were refined with the program REFMAC using the maximum-likelihood option. The final R-factor and free R-factor were 0.212 and 0.279, respectively, for all reflections included within the resolution limits of 15.0 to 1.8 Å.

The final model of MSP119 includes all residues from the amino terminus, Met1, to His91, the second residue of the carboxyterminal hexahistidine tag, with the exception of the segment from Asp66 to Asn68 located at the extremity of a β -hairpin turn. In spite of continuous electron density being present in the region from Asp66 to Asn68, it was not sufficiently well defined to propose a conformation for the main chain of this segment, suggesting that this part of the structure is flexible. Although there was no unambiguous indication for multiple conformers for the side chains of MSP119 in the final electron density maps, the side chains of Lys50, Lys63 and Glu80 could not be modeled beyond their C β atoms because of weak or absent density in these regions. The final model includes 65 solvent molecules. Annex I includes the atomic coordinates which define the *P. cynomolgi* MSP119 3-dimensional molecular structure and Annexes II and III respectively are those of *P. vivax* and *P. falciparum* as determined by homology modeling.

Figure 11A shows the trace of the alpha-carbon backbone and disulfide bridges (bold lines) of the MSP119 molecule from *Plasmodium cynomolgi* determined by the solution of its crystal structure. The two epidermal growth factor (EGF)-like domains predicted from the primary structure are clearly visible as well as the characteristic disulfide bonds which are essential for the maintenance of this complex structure. It is important to note that although only 2 of the 3 "classic" EGF disulfide bonds are present in the first EGF domain for the *P. cynomolgi* and *P. vivax* moieties, the conformation of this domain does not differ significantly from either the second MSP119 EGF domain or other examples of EGF domains with 3 disulfide bridges.

One unexpected feature of the molecular structure of MSP119 is that the 2 EGF-like domains have close and extensive contacts at their interface which include a number of hydrophobic and polar interactions. An important implication of this observation is that the two EGF domains together form a very defined entity which is likely to be essential for the nature of the active protective principle in recombinant analogs of this molecule. These data suggest that the single EGF domains either alone or combined separately would not display the same required conformation.

The probable structures of the corresponding MSP119 antigens derived from *Plasmodium vivax* and *Plasmodium falciparum* (Figures 11B and 11C respectively) were determined by molecular modeling based on the replacement of alternative residues present in the latter two species (11/89 for *P. vivax* and 48/89 + a 4-residue insertion for *P. falciparum*). It is important to note that these replacements do not put any noticeable steric strains on the experimentally determined *P. cynomolgi* MSP119 structure and that the interactions at the EGF-domain interface are maintained by invariant residues or conservative substitutions, in spite of several replacements seen particularly for the *P. falciparum* protein.

It should be emphasized here that, apart from allowing the precise determination of its molecular structure, the crystallization of the MSP119 recombinant protein is *prima facie* evidence of its absolute purity, its quantitatively reproducible, defined conformation as produced in the baculovirus expression system, and its stability. The fact that an identical preparation of *P. cynomolgi* MSP119 recombinant protein (as determined by mass spectrometry) was recently shown to confer excellent protection in the *P. cynomolgi*-toque monkey system described previously (Perera et al., 1998; S. Longacre, I. Holm, L. Perera and S. Handunetti, unpublished data) suggests that this particular conformation is important for protective efficacy.

15 (II) Nuclear magnetic resonance (NMR) "fingerprint" spectra

The three baculovirus MSP119 recombinant proteins derived from *P. cynomolgi*, *P. vivax* and *P. falciparum*, each with a carboxyterminal hexahistidine tag, were purified by metalloaffinity chromatography as described above, dialyzed extensively against 10 mM ammonium bicarbonate, and lyophilized. Five mg of lyophilized protein was dissolved in 380 ml of 20 mM deuterated sodium acetate, 10% D₂O, pH 4.0, centrifuged 30 min at 20°C and loaded into Shigemitsu (Shigemitsu Inc., Allison Park, P.A.) tubes.

In order to obtain an NMR "fingerprint" of a protein, the latter must be pure to at least ca. 95%, as the signals of any proton-containing contaminant present at substantial concentrations would be observed in ¹H NMR spectra. The purity and sequence homogeneity of the MSP119 samples from *P. cynomolgi*, *P. falciparum* and *P. vivax* were assessed by electrospray mass spectrometry. The lyophilized protein was dissolved in

water:methanol:formic acid (50:50:10). The sample was introduced in an API 365 triple-quadrupole mass spectrometer (Perkin Elmer-Sciex, Thornill, Canada) at 5 ml/min by means of a syringe pump (Harvard Apparatus, South Natick, MA). The device was equipped with an atmospheric pressure ion source used to sample positive ions which were produced by a pneumatically-assisted electrospray interface. The ion spray probe tip was held at 4.5 kV and the orifice voltage was set at 45 V. The mass spectrometer was scanned continuously from m/z 950 to 1600 (*P. cynomolgi* sample) or from 1050 to 1900 (*P. falciparum* sample) or from 1150 to 1800 (*P. vivax* sample) with a scan step of 0.1 and a dwell time per step of 2.0 ms. This resulted in a scan duration of 13.0 s (*P. cynomolgi* and *P. vivax* samples) and 17.0 s (*P. falciparum* sample). Ten scans were averaged for each experiment. Mass calibration of the instrument was accomplished by matching propylene glycol ions to their known reference masses which are stored in the mass calibration table of the spectrometer. Data were collected on a Power Macintosh 8600/200 and processed with the Biotoobox 2.2 software from Sciex. The reconstructed mass spectra and m/z spectra of MSP119 from *P. cynomolgi*, *P. falciparum* and *P. vivax* are shown in Figures 12A and B, 12C and D, and 12E and F respectively. The spectra of all three proteins over a wide range of m/z values indicate that there is mainly a single species with no major contaminants, as all the major peaks correspond to different m/z values of a single species or its adducts. The average mass calculated from the experiment for the *P. cynomolgi* (10767 ± 1.1 Da), *P. vivax* (10524.70 ± 0.48) and *P. falciparum* (11041.1 ± 0.6 Da) proteins correspond, within experimental error, to the expected mass of the oxidized proteins with an N-terminus at MSS (*P. cynomolgi* and *P. vivax*) and ISQ (*P. falciparum*). The minor peak in the *P. vivax* sample with an average mass of 10624.54 ± 0.70 corresponds to the expected mass of the same *P. vivax* protein with an N-

terminus at TMSS. Hence, the protein samples used for NMR experiments are greater than 95% pure as required to obtain an NMR "fingerprint" of the proteins and their conformation.

NMR experiments were carried out on a Varian Unity 500 spectrometer (11.7 T) operating at a proton frequency of 500 MHz and equipped with a 5 mm triple-resonance, z-gradient detection probe. Data were processed on a Sun workstation using the software VNMR 5.3 (Varian Inc., Palo Alto). All 2D proton NMR experiments were acquired in the phase-sensitive mode using the hypercomplex scheme (States et al., 1982). ¹H chemical shifts are referred to DSS (sodium 4,4-dimethylsilapentane sulfonate) used as an external reference.

NOESY (States et al., 1982) and TOCSY (Griesinger et al., 1988) spectra were acquired at 35.0°C with 2048 data points in the direct dimension and 256 t₁ increments. Forty (*P. cynomolgi* NOESY), 48 (*P. cynomolgi* TOCSY), 64 (*P. vivax*) or 48 (*P. falciparum*) transients per t₁ increment were accumulated. Solvent suppression was achieved by using the WATERGATE pulse scheme (Piotto et al., 1992). Mixing times in NOESY and TOCSY were 120 and 70 ms, respectively. The spin lock during the mixing time of the TOCSY experiment was obtained by applying the MLEV-17 pulse sequence (Bax and Davis, 1985). Spectra were recorded using a spectral window of 6000 Hz and a recycling delay of 2.2 s (NOESY) or 2.0 s (TOCSY).

2D experiments were transformed using a low pass-filter centered at the transmitter frequency to reduce the intensity of the residual water signal. The width of the filter was 10 Hz and the number of coefficients to calculate the filter shape were 41. Spectra were apodized with shifted square sine bell functions in both dimensions, and baseline corrected in the direct dimension. The first point of every free induction decay (FID) was multiplied by 0.5 prior to Fourier transformation to reduce drift corrections.

The resolution in the indirect dimension (F1) was increased by forward linear prediction to 512 points, except for the TOCSY spectrum of the *P. vivax* sample. Zero filling to 4096 points in the direct dimension (F2) and to 2048 points in the indirect dimension was performed for each spectrum.

5 The chemical shift of a signal in an NMR spectrum depends on the chemical environment of the hydrogen nucleus that produces the signal. The chemical shift, and thereby the topology or peak pattern of an NMR spectrum, is highly dependent on molecular conformation. The TOCSY experiment gives through-bond connectivities within each amino acid
10 residue of a protein while the NOESY experiment provides dipolar (through-space) connectivities between atoms that are less than 5 Å apart. The intensity of a signal strongly depends on the distance between two atoms, as well as on the dynamics of the molecule. The topology of a NOESY spectrum is thus particularly sensitive to differences in molecular
15 conformation. Together, the TOCSY and NOESY spectra provide a detailed fingerprint of the structure of a given protein in solution.

The relevant regions of the NOESY (a and b) and TOCSY (c) spectra of the recombinant MSP119 from *P. cynomolgi* are shown in **Figure 12.0**. The good dispersion of the signals in both spectra is indicative
20 of a folded protein. The down-field shift of many H resonances (**Fig. 12.0a and c**) relative to the random coil range (~4.1~4.8 ppm) and the presence of strong sequential H_i-NH_{i+1} and weak NH-NH correlations in the NOESY spectrum (**Fig. 12.0b and a**, respectively) are all indicative of β-sheet structures and are consistent with the predominantly β-sheet structure
25 determined for MSP119 by crystallography. The corresponding regions of the NOESY and TOCSY spectra of the recombinant MSP119 proteins from *P. vivax* and *P. falciparum* are displayed in **Figures 12.1 and 12.2**. As in the case of the *P. cynomolgi* protein, the spectra of both proteins show good dispersion of chemical shifts, down-field shifted H signals, strong

sequential Hi-NHi+1 and weak NH-NH correlations indicating that these proteins also have a substantial amount of β structures. As the proteins from *P. cynomolgi* and *P. falciparum* have ca. 50% different residues, a comparison of the NMR spectra of both proteins is not possible. However, the presence of β -sheet structures in *P. falciparum* MSP119 as evidenced by the NMR spectra, and the relatively high sequence identity of the proteins (ca. 48% for *P. cynomolgi* and *P. falciparum*) support their structural homology. The NOESY and TOCSY spectra of the *P. cynomolgi* and *P. vivax* proteins display many crosspeaks as well as crosspeak patterns at the same (or similar) chemical shifts. The similarities that can be observed in the spectra of both proteins, in spite of the existence of 11 different residues, suggest that both proteins have a similar structure. Thus, the NMR data, as well as the high sequence identity of both proteins (ca. 86%), strongly support the similitude of the MSP119 structures from *P. cynomolgi* and *P. vivax* species as postulated by homology modeling based on the crystallographic data.

Unlike X-ray crystallography, NMR technology provides a relatively accessible means of assessing if a protein preparation displays a given conformation. Thus, once having defined in detail a conformation which constitutes the active principle of a protective antigen, one can verify by NMR whether competing preparations contain conformationally similar species and/or whether these represent a mixture of more than one conformer (with an estimated 5-10% limit of detection). It should be noted that some variation in the peak position can be expected for independent experiments performed under the same conditions and with the same protein, with an estimated ± 0.02 ppm variation in each dimension. Also, as the contour plots presented in Figures 12.0, 12.1 and 12.2 are cross-sections of a spectrum which contains three-dimensional peaks as well as noise, and in which peak intensity depends on many experimental and

data-processing factors (such as the sensitivity of the probe, the suppression of the water signal, the phasing of the spectrum and the functions used to apodize the spectrum and correct the baseline) in addition to the nuclei interactions, some variations in the intensity and thereby in the pattern of crosspeaks at a given cross-section level must be expected, especially for the less intense peaks, the peaks near the diagonal and those close to the water signal (4.67 ppm). Bearing this in mind, the NMR spectra can serve as an identifying "fingerprint" which can be used not only for product quality control, but also to establish distinguishing characteristics of different preparations of the same or similar polypeptides.

(III) ELISA analysis of anti-MSP119 and anti-MSP142 antibody reactivity with native and reduced antigen

Recombinant baculovirus *Plasmodium vivax* and *Plasmodium cynomolgi* MSP119 and MSP142 was produced and purified by immunoaffinity or metalloaffinity chromatography as described (Holm et al., 1997; see above). The antisera from *Macaca sinica* (toque) monkeys vaccinated with baculovirus recombinant *P. cynomolgi* MSP119 or MSP142 was obtained as described by Perera et al. (1998). Anti-*P. vivax* human antisera were obtained from endemically exposed donors in Thailand.

For the ELISA assay, microtiter plates (NUNC) were coated with the purified MSP119 or MSP142 recombinant protein at 200 ng ml^{-1} overnight at 4°C . After 3 washes with PBS containing 0.1% Tween 20 (PBS-T), the polyclonal human, monkey and mouse antisera were incubated for 2 h at 37°C . Plates were washed and incubated for 1 h at 37°C with horseradish peroxidase conjugated anti-human or anti-mouse IgG. After 5 washes, 100 μl of freshly prepared 0.2% orthophenylenediamine containing 0.03% hydrogen peroxidase in 0.1 M citrate buffer, pH 5.2 were added to each

well. The reaction was stopped by the addition of 50 μ l of 3 N HCl and the optical density was measured at 492 nm. For ELISA assays done with reduced coating antigen, the antigen was reduced *in situ* by incubation in the microtiter plates with 0.2 M Tris-HCl (pH 8.5), 5 mM EDTA, 20 mM dithiothreitol for 2 h at room temperature followed by alkylation for 20 min in the dark with 60 mM iodoacetamide added to the reducing reaction mixture.

Figures 13 A-F show ELISA titration of tertiary antisera from 8 individual toque monkeys corresponding to 2 groups of 3 animals each vaccinated with either recombinant *P. cynomolgi* MSP119 or MSP142 (Perera et al. 1998). In each figure a given individual antisera is reacted with either the MSP119 or MSP142 antigen in native (N) or reduced, denatured (D) form. In the 3 antisera from MSP119 vaccinated animals (A-C) virtually all the epitopes in the MSP119 or MSP142 (also containing the MSP119 polypeptide) coating antigens are reduction sensitive since no significant ELISA reactivity is observed when the coating antigens are reduced. These results indicate clearly that the macaque humoral immune response to the recombinant MSP119 polypeptide, whether presented alone or in the context of the larger MSP142 protein, is primarily directed against reduction sensitive conformational epitopes. Since these MSP119 vaccinated monkeys were extremely well protected against a challenge infection of the *P. cynomolgi* blood stage parasite (Perera et al., 1998), it is thus highly likely that a conformationally intact recombinant MSP119 antigen is crucial for a relevant, protective immune response. The crystallographic results described above indicate that the MSP119 recombinant antigen obtained from this higher order expression system represents a single conformational species which must constitute, at least in part, the active principle in these vaccine preparations.

The antisera from MSP142 vaccinated monkeys (**Figures 13 D-F**) show that although all the MSP142 epitopes are not reduction sensitive, those corresponding to the MSP119 antigen are completely sensitive. From the above observations regarding the MSP119 vaccinated animals, it is likely that antibodies specific for the conformationally sensitive epitopes were important for the good protection conferred by the MSP142 antigen in 2 of the 3 vaccinated monkeys (n° 434 and 428; Perera et al., 1998). Nevertheless, the third animal (n° 435), which also had reduction sensitive anti-MSP119 antibodies, was not protected and it is possible that in some cases antibodies specific for non-conformational epitopes in the MSP142 immunogen could interfere with protection, arguing against the use of the MSP142 antigen in vaccine preparations.

Figure 14 shows the recombinant MSP119 reactivity of antisera (1:100 dilution) from 22 *P. vivax* infected Thai donors as determined by ELISA analysis using native or reduced antigen. The data from **Figures 14A and 14B** were obtained using immunoaffinity or metalloaffinity purified antigens respectively. In all cases the native antigen is much more reactive than the reduced moiety, indicating that the large majority of MSP119 epitopes recognized by human anti-parasite sera are reduction sensitive. Thus any protective effect, as well as natural boosting, of an MSP119 based malaria vaccine would likely depend on the quantitative presence of such conformational epitopes in the vaccinating immunogen.

For **Figure 15**, 50 and 25 mg respectively of recombinant *P. cynomolgi* MSP142 and MSP119 purified by immunoaffinity chromatography (Holm et al., 1997) were inoculated subcutaneously three times into mice at 10 to 14 day intervals in the absence of adjuvant. A fourth and final boost was administered 8 months after the third injection. The dependence of the humoral immune response on reduction sensitive

conformational epitopes present in the recombinant antigens was analyzed with pooled anti-MSP142 or anti-MSP119 sera using the ELISA technique with native or reduced antigen as described above.

The results show that while the anti-MSP142 response is partially dependent on reduction sensitive conformational epitopes in the p42 moiety after 3 closely spaced injections (Third bleed), this dependence appears to be significantly reduced in the antisera obtained by boosting after an 8 month interval (Fourth bleed). Since the MSP142 antisera reactivity directed against MSP119 is completely dependent on reduction sensitive epitopes, the long term memory response to the MSP142 antigen in the absence of adjuvant clearly favors non-conformational, non-MSP119 epitopes. In contrast, the anti-MSP119 sera are completely dependent on reduction sensitive conformational epitopes regardless of the short or long term memory context. These observations, although preliminary, suggest that the N-terminal, non-p19 epitopes in the MSP142 might be favored by natural boosting to the detriment of the conformational MSP119 epitopes which appear to be important for protection (see above). This would also discourage the use of the MSP142 for vaccination under conditions where natural boosting by antigens containing MSP142 specific epitopes is expected.

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ANNEX I

57

REMARK Written by O version 5.9.1

REMARK Wed May 13 11:19:38 1998

CRYST1 43.770 43.770 92.040 90.00 90.00 120.00

ORIGX1 1.000000 0.000000 0.000000 0.000000

ORIGX2 0.000000 1.000000 0.000000 0.000000

ORIGX3 0.000000 0.000000 1.000000 0.000000

SCALE1 0.022847 0.013189 -0.000001 0.000000

SCALE2 0.000000 0.026381 -0.000001 0.000000

SCALE3 0.000000 0.000000 0.010865 0.000000

REMARK	Atom	Residue no.	X	Y	Z	Occ.	B-factor	Z
ATOM	1	N MET	1	1.213	13.847	37.031	1.00 51.49	7
ATOM	2	CA MET	1	-0.202	14.188	36.715	1.00 51.41	6
ATOM	3	C MET	1	-0.492	14.176	35.214	1.00 49.92	6
ATOM	4	O MET	1	0.358	14.544	34.406	1.00 49.90	8
ATOM	5	CB MET	1	-0.532	15.576	37.269	1.00 54.35	6
ATOM	6	CG MET	1	-2.020	15.889	37.300	1.00 54.84	6
ATOM	7	SD MET	1	-2.345	17.262	38.417	1.00 60.93	16
ATOM	8	CE MET	1	-1.912	18.676	37.414	1.00 53.34	6
ATOM	9	N SER	2	-1.730	13.837	34.870	1.00 46.47	7
ATOM	10	CA SER	2	-2.160	13.819	33.477	1.00 44.34	6
ATOM	11	C SER	2	-2.177	15.233	32.909	1.00 42.35	6
ATOM	12	O SER	2	-2.584	16.178	33.584	1.00 38.52	8
ATOM	13	CB SER	2	-3.526	13.156	33.364	1.00 47.86	6
ATOM	14	OG SER	2	-4.433	13.932	32.606	1.00 51.95	8
ATOM	15	N SER	3	-1.742	15.363	31.656	1.00 37.95	7
ATOM	16	CA SER	3	-1.537	16.657	31.033	1.00 33.60	6
ATOM	17	C SER	3	-2.810	17.465	30.900	1.00 27.50	6
ATOM	18	O SER	3	-2.767	18.695	30.941	1.00 27.48	8
ATOM	19	CB SER	3	-0.863	16.486	29.653	1.00 37.21	6
ATOM	20	OG SER	3	-1.485	17.363	28.726	1.00 40.23	8
ATOM	21	N GLU	4	-3.957	16.826	30.773	1.00 26.43	7
ATOM	22	CA GLU	4	-5.229	17.497	30.655	1.00 24.35	6
ATOM	23	C GLU	4	-5.537	18.349	31.905	1.00 22.72	6
ATOM	24	O GLU	4	-6.288	19.309	31.806	1.00 20.76	8
ATOM	25	CB GLU	4	-6.381	16.483	30.627	1.00 32.86	6
ATOM	26	CG GLU	4	-6.937	16.258	29.223	1.00 39.74	6
ATOM	27	CD GLU	4	-6.251	15.048	28.604	1.00 39.44	6
ATOM	28	OE1 GLU	4	-5.388	14.455	29.288	1.00 43.47	8
ATOM	29	OE2 GLU	4	-6.569	14.710	27.457	1.00 35.55	8
ATOM	30	N HIS	5	-5.018	17.863	33.042	1.00 20.20	7
ATOM	31	CA HIS	5	-5.365	18.609	34.277	1.00 18.01	6
ATOM	32	C HIS	5	-4.302	19.574	34.743	1.00 21.28	6
ATOM	33	O HIS	5	-4.466	20.228	35.796	1.00 19.19	8
ATOM	34	CB HIS	5	-5.686	17.574	35.369	1.00 17.23	6
ATOM	35	CG HIS	5	-6.924	16.767	35.111	1.00 16.21	6
ATOM	36	ND1 HIS	5	-8.204	17.256	35.259	1.00 18.48	7
ATOM	37	CD2 HIS	5	-7.037	16.467	34.737	1.00 16.39	6
ATOM	38	CE1 HIS	5	-9.066	16.286	34.989	1.00 19.00	6

ANNEX II

58

REMARK Written by O version 5.9.1

REMARK Fri Jul 10 10:11:56 1998

CRYST1	43.770	43.770	92.040	90.00	90.00	120.00
ORIGX1	1.000000	0.000000	0.000000			0.000000
ORIGX2	0.000000	1.000000	0.000000			0.000000
ORIGX3	0.000000	0.000000	1.000000			0.000000
SCALE1	0.022847	0.013189	-0.000001			0.000000
SCALE2	0.000000	0.026381	-0.000001			0.000000
SCALE3	0.000000	0.000000	0.010865			0.000000

REMARK	Atom	Residue no.	X	Y	Z	Occ.	B-factor	Z
ATOM	1	N MET	1	1.213	13.847	37.031	1.00 51.49	7
ATOM	2	CA MET	1	-0.202	14.188	36.715	1.00 51.41	6
ATOM	3	C MET	1	-0.492	14.176	35.214	1.00 49.92	6
ATOM	4	O MET	1	0.358	14.544	34.406	1.00 49.90	8
ATOM	5	CB MET	1	-0.532	15.576	37.269	1.00 54.35	6
ATOM	6	CG MET	1	-2.020	15.889	37.300	1.00 54.84	6
ATOM	7	SD MET	1	-2.345	17.262	38.417	1.00 60.93	16
ATOM	8	CE MET	1	-1.912	18.676	37.414	1.00 53.34	6
ATOM	9	N SER	2	-1.730	13.837	34.870	1.00 46.47	7
ATOM	10	CA SER	2	-2.160	13.819	33.477	1.00 44.34	6
ATOM	11	C SER	2	-2.177	15.233	32.909	1.00 42.35	6
ATOM	12	O SER	2	-2.584	16.178	33.584	1.00 38.52	8
ATOM	13	CB SER	2	-3.526	13.156	33.364	1.00 47.86	6
ATOM	14	OG SER	2	-4.433	13.932	32.606	1.00 51.95	8
ATOM	15	N SER	3	-1.742	15.363	31.656	1.00 37.95	7
ATOM	16	CA SER	3	-1.537	16.657	31.033	1.00 33.60	6
ATOM	17	C SER	3	-2.810	17.465	30.900	1.00 27.50	6
ATOM	18	O SER	3	-2.767	18.695	30.941	1.00 27.48	8
ATOM	19	CB SER	3	-0.863	16.486	29.653	1.00 37.21	6
ATOM	20	OG SER	3	-1.485	17.363	28.726	1.00 40.23	8
ATOM	21	N GLU	4	-3.957	16.826	30.773	1.00 26.43	7
ATOM	22	CA GLU	4	-5.229	17.497	30.655	1.00 24.35	6
ATOM	23	C GLU	4	-5.537	18.349	31.905	1.00 22.72	6
ATOM	24	O GLU	4	-6.288	19.309	31.806	1.00 20.76	8
ATOM	25	CB GLU	4	-6.381	16.483	30.627	1.00 32.86	6
ATOM	26	CG GLU	4	-6.937	16.258	29.223	1.00 39.74	6
ATOM	27	CD GLU	4	-6.251	15.048	28.604	1.00 39.44	6
ATOM	28	OE1 GLU	4	-5.388	14.455	29.288	1.00 43.47	8
ATOM	29	OE2 GLU	4	-6.569	14.710	27.457	1.00 35.55	8
ATOM	30	N HIS	5	-5.018	17.863	33.042	1.00 20.20	7
ATOM	31	CA HIS	5	-5.365	18.609	34.277	1.00 18.01	6
ATOM	32	C HIS	5	-4.302	19.574	34.743	1.00 21.28	6
ATOM	33	O HIS	5	-4.466	20.228	35.796	1.00 19.19	8
ATOM	34	CB HIS	5	-5.686	17.574	35.369	1.00 17.23	6
ATOM	35	CG HIS	5	-6.924	16.767	35.111	1.00 16.21	6
ATOM	36	ND1 HIS	5	-8.204	17.256	35.259	1.00 18.48	7
ATOM	37	CD2 HIS	5	-7.037	15.467	34.737	1.00 16.39	6
ATOM	38	CE1 HIS	5	-9.066	16.286	34.989	1.00 19.00	6

ANNEX III

59

REMARK Written by O version 5.9.1

REMARK Wed May 13 12:12:21 1998

CRYST1	43.770	43.770	92.040	90.00	90.00	120.00
ORIGX1	1.000000	0.000000	0.000000			0.000000
ORIGX2	0.000000	1.000000	0.000000			0.000000
ORIGX3	0.000000	0.000000	1.000000			0.000000
SCALE1	0.022847	0.013189	-0.000001			0.000000
SCALE2	0.000000	0.026381	-0.000001			0.000000
SCALE3	0.000000	0.000000	0.010865			0.000000

REMARK	Atom	Residue no.	X	Y	Z	Occ.	B-factor	Z
ATOM	1	N ASN	1	1.196	13.850	37.004	1.00 51.49	7
ATOM	2	CA ASN	1	-0.208	14.191	36.732	1.00 51.41	6
ATOM	3	C ASN	1	-0.469	14.171	35.223	1.00 49.92	6
ATOM	4	O ASN	1	0.358	14.544	34.406	1.00 49.90	8
ATOM	5	CB ASN	1	-0.523	15.583	37.280	1.00 54.35	6
ATOM	6	CG ASN	1	0.685	16.519	37.262	1.00 54.84	6
ATOM	7	OD1 ASN	1	1.428	16.583	38.240	1.00 20.00	8
ATOM	8	ND2 ASN	1	0.930	17.259	36.196	1.00 20.00	7
ATOM	9	N ILE	2	-1.728	13.838	34.876	1.00 46.47	7
ATOM	10	CA ILE	2	-2.162	13.813	33.472	1.00 44.34	6
ATOM	11	C ILE	2	-2.177	15.238	32.908	1.00 42.35	6
ATOM	12	O ILE	2	-2.584	16.178	33.584	1.00 38.52	8
ATOM	13	CB ILE	2	-3.559	13.197	33.374	1.00 47.86	6
ATOM	14	CG1 ILE	2	-3.621	11.765	33.903	1.00 20.00	6
ATOM	15	CG2 ILE	2	-4.082	13.133	31.937	1.00 20.00	6
ATOM	16	CD1 ILE	2	-5.017	11.149	33.796	1.00 20.00	6
ATOM	17	N SER	3	-1.742	15.363	31.656	1.00 37.95	7
ATOM	18	CA SER	3	-1.537	16.657	31.033	1.00 33.60	6
ATOM	19	C SER	3	-2.810	17.465	30.900	1.00 27.50	6
ATOM	20	O SER	3	-2.767	18.695	30.941	1.00 27.48	8
ATOM	21	CB SER	3	-0.863	16.486	29.653	1.00 37.21	6
ATOM	22	OG SER	3	-1.485	17.363	28.726	1.00 40.23	8
ATOM	23	N GLN	4	-3.949	16.826	30.782	1.00 26.43	7
ATOM	24	CA GLN	4	-5.246	17.504	30.650	1.00 24.35	6
ATOM	25	C GLN	4	-5.528	18.342	31.901	1.00 22.72	6
ATOM	26	O GLN	4	-6.288	19.309	31.806	1.00 20.76	8
ATOM	27	CB GLN	4	-6.361	16.472	30.475	1.00 32.86	6
ATOM	28	CG GLN	4	-7.128	16.639	29.163	1.00 39.74	6
ATOM	29	CD GLN	4	-8.236	15.600	28.984	1.00 39.44	6
ATOM	30	OE1 GLN	4	-7.994	14.534	28.421	1.00 43.47	8
ATOM	31	NE2 GLN	4	-9.452	15.848	29.433	1.00 20.00	7
ATOM	32	N HIS	5	-5.018	17.863	33.042	1.00 20.20	7
ATOM	33	CA HIS	5	-5.365	18.609	34.277	1.00 18.01	6
ATOM	34	C HIS	5	-4.302	19.574	34.743	1.00 21.28	6
ATOM	35	O HIS	5	-4.466	20.228	35.796	1.00 19.19	8
ATOM	36	CB HIS	5	-5.686	17.574	35.369	1.00 17.23	6
ATOM	37	CG HIS	5	-5.924	16.767	35.111	1.00 16.25	6
ATOM	38	ND1 HIS	5	-8.204	17.256	35.259	1.00 18.48	7

CLAIMS

1. A recombinant protein whose essential constituent polypeptide sequence comprises:

- a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite other than *Plasmodium vivax* which is infectious in man, the C-terminal fragment remaining normally anchored to the surface of the surface of the parasite at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
- or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit an *in vivo* parasitemia in a host infected with such parasite;
- or that of a peptide which is capable of inducing a cellular and/or humoral immunological response equivalent to that produced by said p19 fragment or said portion of that fragment; and

wherein said recombinant protein comprises conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognized by human antisera formed against the corresponding *Plasmodium*.

2. The recombinant protein of claim 1 which is not recognized by said human antisera when it is in the reduced form.

3. The recombinant protein of claim 2 which is substantially free of any form of a recombinant protein having the same sequence of amino acids, yet in which the conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognized by human antisera formed against the corresponding *Plasmodium* are not in a conformational form as defined by:

(a) the atomic coordinates as detailed in Annexes I, II or II, obtained by crystallography; and

- (b) the NMR fingerprints as illustrated in Figures 12.0a to 12.2c.
4. The recombinant protein of claim 2 in which at least 20% contains an epitope in a conformational form as defined by the NMR fingerprints as illustrated in Figures 12.0a to 12.2c.
 - 5 5. The recombinant protein of claim 2 or claim 3 which is in a pure state.
 6. A recombinant protein of claim 1, which is recognized by human antisera formed against the corresponding *Plasmodium* or against a homologous *Plasmodium* when it is in its non reduced state or in a reduced non irreversible state, but is not recognized or is only recognized to a slight extent by these same antisera when it is irreversibly reduced.
 - 10 7. The recombinant protein of claim 5 which is defined by the crystallized form with the atomic coordinates as detailed in Annexes I, II or III, and the NMR fingerprints as illustrated in Figures 12.0a to 12.2c.
 - 15 8. The recombinant protein of claim 5 which has a very high degree of purity as determined by electrospray mass spectrometry.
 9. The recombinant protein of claim 5 which elicits a long term memory response directed in a substantially specific manner against said conformational epitopes in animals to which they are administered.
 - 20 10. The protein of claim 1 which inhibits the reactivity of an immune antiserum against p42 produced from the same MSP-1 protein and is itself only partially inhibited by an immune antiserum produced against p42.
 - 25 11. The recombinant of claim 1 which is essentially free of any polypeptide having a sequence of amino acids in the C-terminal region of p33 (33 kDa N-terminal fragment) resulting from the natural cleavage of the p42 protein of the same MSP1 protein.

12. The recombinant protein of claim 1 which comprises, upstream of the polypeptide sequence of p19, a polypeptide region whose sequence is the C-terminal region of p33 resulting from the cleavage of p42 of the same MSP-1 protein, wherein said polypeptide region contains less than 50 amino acid residues.
13. The recombinant protein of claim 11 wherein said polypeptide region contains less than 10 amino acid residues.
14. The recombinant protein of claim 1 which comprises, upstream of the polypeptide sequence of p19, a polypeptide region whose sequence is the C-terminal region of the p33 resulting from the cleavage of p42 of the same MSP-1 protein, wherein said C-terminal portion is restricted to that part which is substantially conserved in *P. falciparum* and *P. vivax*.
15. The recombinant protein of claim 1 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
16. The recombinant protein of claim 2 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.
17. The recombinant protein of claim 3 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.
18. The recombinant protein of claim 4 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.
19. The recombinant protein of claim 5 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.

20. The recombinant protein of claim 6 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.
21. The recombinant protein of claim 7 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.
22. The recombinant protein of claim 8 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.
23. The recombinant protein of claim 9 wherein the sequence of amino acids of said essential constituent polypeptide encompasses the two sequences of the two EGF regions of the p19 protein.
24. The recombinant protein according to claim 1, wherein the constituent polypeptide carries a glycosylphosphatidylinositol (GPI) group of the type which enables the p19 fragment to anchor to the membrane of a eukaryotic cell infected with the MSP-1 protein.
25. The recombinant protein of claim 1, wherein the constituent polypeptide is free of the sequence of amino acids in the hydrophobic C-terminal portion of the p19 which intervenes in the induction of an anchoring of said p19 to the cell membrane of a host infected with a *Plasmodium* type parasite.
26. The recombinant protein according to claim 24, which is hydrosoluble.
27. The recombinant protein of claim 1 which comprises the sequences of amino acids of the p19 of the MSP-1 protein of *Plasmodium falciparum*.
28. The recombinant protein of claim 1 which comprises the sequence of amino acids of the MSP-1 protein of *Plasmodium cynomolgi*.
29. An oligomer of the recombinant protein of claim 1.

30. The oligomer of claim 29, which comprises from 2 to 50 monomer units of the sequence of said recombinant protein;
31. The recombinant protein of claim 1, which is conjugated to a carrier molecule for use in the production of vaccines.
- 5 32. A vaccination composition against a *Plasmodium* type parasite which is infectious in man, containing as an active principle a recombinant protein whose essential constituent polypeptide sequence comprises:
- a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious in man, said C-terminal fragment remaining normally anchored to the surface of the parasite at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
 - 10 • or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit an *in vivo* parasitemia in a host infected with such parasite;
 - 15 • or that of a peptide which is capable of inducing a cellular and/or humoral immunological response equivalent to that produced by said p19 fragment or said portion of that fragment; and
 - 20 said recombinant protein further comprising conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognized by human antisera formed against the corresponding *Plasmodium*.
- 25 33. The vaccinating composition of claim 32, wherein said recombinant protein is not recognized by said human antisera when it is in the reduced form.
34. The vaccinating composition of claim 32, wherein said recombinant protein is substantially free of any form of said recombinant protein

having the same sequence of amino acids, yet in which the conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognized by human antisera formed against the corresponding *Plasmodium* are not in the conformational form as defined by:

- (a) the atomic coordinates as detailed in Annexes I, II or II, obtained by crystallography; and
- (b) the NMR fingerprints as illustrated in Figures 12.0a to 12.2c.

- 35. The vaccinating composition of claim 32, wherein said recombinant protein is in a pure state.
- 36. The vaccinating composition of claim 32, wherein said recombinant protein is recognized by human antisera formed against the corresponding *Plasmodium* or against a homologous *Plasmodium* when it is in its non reduced state or in a reduced non irreversible state, but is not recognized or is only recognized to a slight extent by these same antisera when it is irreversibly reduced.
- 37. The vaccinating composition of claim 32, wherein said recombinant protein is defined by the crystallized form with the atomic coordinates as detailed in Annexes I, II or III, and the NMR fingerprints as illustrated in Figures 12.0a to 12.2c.
- 38. The vaccinating composition of claim 32, wherein said recombinant protein has a very high degree of purity as determined by electrospray mass spectroscopy.
- 39. The vaccinating composition of claim 32, wherein said recombinant protein elicits a long term memory response directed in a substantially specific manner against said conformational epitopes in animals to which they are administered.

40. The vaccinating composition of claim 32, wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
41. The vaccinating composition of claim 33, wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
42. The vaccinating composition of claim 34, wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
43. The vaccinating composition of claim 35, wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
44. The vaccinating composition of claim 36, wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
45. The vaccinating composition of claim 37, wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
46. The vaccinating composition of claim 38, wherein the sequence of amino acids of said essential constituent polypeptide encompasses the sequences of the two EGF regions of the p19 protein.
47. The vaccinating composition of claim 32 wherein the essential constituent polypeptide of said recombinant protein comprises the sequence of the p19 *Plasmodium falciparum*.
48. The vaccinating composition of claim 32 wherein the essential constituent polypeptide of said recombinant protein comprises the sequence of the p19 *Plasmodium vivax*.
49. An antibody which specifically recognizing the p19 of a MSP-1 protein of the merozoite form of a *Plasmodium* type parasite which is

infectious in man other than *Plasmodium vivax* and which does not recognize *Plasmodium vivax*.

50. The antibody of claim 49, which is monoclonal.

51. The monoclonal antibody of claim 50, which specifically recognizes the p19 of *P. falciparum*.

52. The monoclonal antibody of claim 50, which specifically recognizes the p19 of *P. vivax*.

53. A recombinant baculovirus type modified vector containing, under the control of a promoter contained in the vector and able to be recognized by cells transfectable by said vector, a first nucleotide sequence coding for a signal peptide and a second nucleotide sequence downstream of the first nucleotide sequence, also under the control of said promoter, wherein said first nucleotide sequence authorizes the expression of said second nucleotide sequence in a baculovirus system and wherein said second nucleotide sequence codes for one of the following peptide sequences:

- a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious in man, the C-terminal fragment remaining normally anchored to the surface of the parasite at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
- or of a portion of that peptide fragment provided that the expression product from the second sequence in a baculovirus system is also capable of inducing an immune response which can inhibit an *in vivo* parasitemia in a host infected with said parasite;

- or of a peptide which is capable of inducing a cellular or humoral immunological response equivalent to that produced by said peptide fragment p19 or said peptide fragment portion; and wherein said second nucleotide sequence has a G and C content in the range of from 40% to 60% of the totality of nucleotides from which said second nucleotide sequence is constituted.
- 54. The vector of claim 53, wherein said second nucleotide sequence has a G and C content in the range of at least 50% of the totality of nucleotides from which said second nucleotide sequence is constituted.
- 55. The vector of claim 53, wherein the second nucleotide sequence is a synthetic sequence.
- 56. The vector of claim 53, wherein the first nucleotide sequence codes for a signal peptide from *Plasmodium vivax* or *Plasmodium falciparum* normally associated with the *Plasmodium* MSP-1 protein.
- 57. The vector of claim 53, which consists of a modified baculovirus.
- 58. An organism transfected by the vector of claim 53.
- 59. A synthetic DNA containing a first nucleotide sequence including a portion which codes for the peptide sequence:
 - of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of *Plasmodium falciparum*, said C-terminal fragment remaining normally anchored to the surface of the parasite at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
 - or of a portion of that peptide fragment provided that the expression product of said DNA in a baculovirus system is also capable of inducing an immune response which can inhibit an *in vivo* parasitemia in a host infected with such parasite;

- or of a peptide capable of inducing a cellular or humoral type immunological response equivalent to that produced by said p19 peptide fragment or said portion of that fragment or both; and wherein said nucleotide sequence has a G and C nucleotide content in the range of from 40% to 60% of the totality of nucleotides from which said synthetic DNA is constituted.
60. The synthetic DNA of claim 59, wherein said nucleotide sequence has a G and C content of at least 50% of the totality of nucleotides from which said synthetic DNA is constituted.
61. The synthetic DNA sequence of claim 59, which further comprises upstream from said first nucleotide sequence, a second nucleotide sequence coding for a signal peptide normally associated with a *Plasmodium* MSP-1 protein which is homologous or heterologous relative to said first nucleotide sequence.
62. The synthetic DNA sequence of claim 61, wherein the signal sequence originates from *P. vivax*.
63. A baculovirus type selected from the group comprising:
- a virus deposited at the CNCM [Collection Nationale de Cultures de Microorganismes; National Collection of Microorganism Cultures] with registration number I-1659;
 - a virus deposited at the CNCM with registration number I-1660;
 - a virus deposited at the CNCM with registration number I-1661;
 - a virus deposited at the CNCM with registration number I-1662;
 - a virus deposited at the CNCM with registration number I-1663; and
 - a virus deposited at the CNCM with registration number I-2041.
64. A hybridoma secreting the monoclonal antibodies of claim 50.
65. A hybridoma secreting the monoclonal antibodies of claim 51.

66. A hybridoma which secretes the monoclonal antibody of claim 50, which has been deposited at the CNCM, (Paris, France) with registration number I-1846, on the 14th of February 1997.

67. A vaccine composition according to claim 32, which comprises a mixture of nucleotide sequences selected from the mixtures of recombinant proteins comprising the sequences of:

- *P. falciparum* p19 and *P. vivax* p19;
- *P. falciparum* p19 and *P. falciparum* p42;
- *P. vivax* p19 and *P. vivax* p42;
- *P. falciparum* p19 and *P. falciparum* p42; and
P. vivax p19 and *P. vivax* p42.

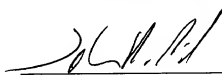
REQUEST FOR RECONSIDERATION

Applicants have amended the claims to correct formal matters with respect to language and multiple dependencies. No new matter has been added. Claims 1-67 remain pending in this application. Favorable consideration is respectfully requested.

Applicants respectfully submit that the above-identified application is now in condition for examination on the merits and early notice of such action is earnestly solicited.

Respectfully submitted,

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Shirley LONGACRE-ANDRE et al.

FOR: RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF PLASMODIUM
MSP-1

FILED: Herewith

LETTER

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Listed below are the names and addresses of the inventors for the above-identified patent application.

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A declaration containing all the necessary information will be submitted at a later date.

Respectfully submitted,

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FIGURE 1A

		E	F	N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E	N
Bac 19		<u>GAA</u>	<u>TTT</u>	AAC	ATC	TCG	CAG	CAC	CAA	TGC	GTG	AAA	AAA	CAA	TGT	CCC	GAG	AAC
PF 19																		
		AAC	ATT	TCA	CAA	CAC	CAA	TGC	GTA	AAA	AAA	CAA	TGT	CCA	GAA	AAT		

		S	G	C	F	R	H	L	D	E	R	E	E	C	K	C	L	L
Bac 19		TCT	GGC	TGT	TTC	AGA	CAC	TTG	GAC	GAG	AGA	GAG	GAG	TGT	AAA	TGT	CTG	CTG
PF 19																		
		TCT	GGA	TGT	TTC	AGA	CAT	TTA	GAT	GAA	AGA	GAA	GAA	TGT	AAA	TGT	TTA	TTA

		N	Y	K	Q	E	G	D	K	C	V	E	N	P	N	P	T	C
Bac 19		AAC	TAC	AAA	CAG	GAG	GGC	AAC	AAG	TGC	GTG	GAG	AAC	CCC	AAC	CCG	ACC	TGT
PF 19																		
		AAT	TAC	AAA	CAA	GAA	GCT	GAT	AAA	TGT	GTT	GAA	AAT	CCA	AAT	CCT	ACT	TGT

		N	E	N	N	G	G	C	D	A	D	A	K	C	T	E	E	D
Bac 19		AAC	GAG	AAC	AAC	GGC	GGC	TGT	GAC	GCA	GAC	GCC	AAA	TGC	ACC	GAG	GAG	GAC
PF 19																		
		AAC	GAA	AAT	AAT	GGT	GGA	TGT	GAT	GCA	GAT	GCC	AAA	TGT	ACC	GAA	GAA	GAT

		S	G	S	N	G	K	K	I	T	C	E	C	T	K	P	D	S
Bac 19		TCG	GGC	AGC	AAC	GGC	AAG	AAA	ATC	ACG	TGT	GAG	TGT	ACC	AAA	CCC	GAC	TCG
PF 19																		
		TCA	GGT	AGC	AAC	GGA	AAG	AAA	ATC	ACA	TGT	GAA	TGT	ACT	AAA	CCT	GAT	TCT

		Y	P	L	F	D	G	I	F	C	S	*	*
Bac 19		TAC	CCG	CTG	TTC	GAC	GGC	ATC	TTC	TGC	AGC	TAA	TAA
PF 19													
		TAT	CCA	CTT	TTC	GAT	GGT	ATT	TTC	TGC	AGT		

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FIGURE 1B

	Site Eco RI																			
	E	F	N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E	N			
Bac 19	GAG	TTT	AAC	ATC	TCG	CAG	CAC	CAA	TGC	GTG	AAA	AAA	CAA	TGT	CCC	GAG	AAC			
PF 19			AAC	ACT	TCA	CAA	CAC	CAA	TGC	GTA	AAA	AAA	CAA	TGT	CCA	GAA	AAT			
	S	G	C	F	R	H	L	D	E	R	E	E	C	K	C	L	L			
Bac 19	TCT	GGC	TGT	TTC	AGA	CAC	TTG	GAC	GAG	AGA	GAG	GAG	TGT	AAA	TGT	CTG	CTG			
PF 19	TCT	GGA	TGT	TTC	AGA	CAT	TTA	GAT	GAA	AGA	GAA	GAA	TGT	AAA	TGT	TTA	TTA			
	N	Y	K	Q	E	G	D	K	C	V	E	N	P	N	P	T	C			
Bac 19	AAC	TAC	AAA	CAG	GAG	GGC	GAC	AAG	TGC	GTG	GAG	AAC	CCC	AAC	CCG	ACC	TGT			
PF 19	AAT	TAC	AAA	CAA	GAA	GGT	GAT	AAA	TGT	GTT	GAA	AAT	CCA	AAT	CCT	ACT	TGT			
	N	E	N	N	G	G	C	D	A	D	A	K	C	T	E	E	D			
Bac 19	AAC	GAG	AAC	AAC	GGC	GGC	TGT	GAC	GCA	GAC	GGC	AAA	TGC	ACC	GAG	GAG	GAC			
PF 19	AAC	GAA	AAT	AAT	GGT	GGA	TGT	GAT	GCA	GAT	GGC	AAA	TGT	ACC	GAA	GAA	GAT			
	S	G	S	N	G	K	K	I	T	C	E	C	T	K	P	D	S			
Bac 19	TCG	GGC	AGC	AAC	GGC	AAG	AAA	ATC	ACG	TGT	GAG	TGT	ACC	AAA	CCC	GAC	TCG			
PF 19	TCA	GGT	AGC	AAC	GGA	AAG	AAA	ATC	ACA	TGT	GAA	TGT	ACT	AAA	CCT	GAT	TCT			
	Y	P	L	F	D	G	I	F	C	S	S	S	N	F	L	G	I			
Bac 19	TAC	CCG	CTG	TTC	GAC	GGC	ATC	TTC	TGC	AGC	TCC	TCT	AAC	TTC	TTG	GGC	ATC			
PF 19	TAT	CCA	CTT	TTC	GAT	GGT	ATT	TTC	TGC	AGT	TCC	TCT	AAC	TTC	TTA	GGA	ATA			
	S	F	L	L	I	L	M	L	I	L	Y	S	F	I	*	*				
Bac 19	TCG	TTC	TTG	TTG	ATC	CTC	ATG	TTG	ATC	TTG	TAC	AGC	TTC	ATT	TAA	TAA				
PF 19	TCA	TTC	TTA	TTA	ATA	CTC	ATG	TTA	ATA	TTA	TAC	AGT	TTC	ATT						

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ATG	AAG	GCG	CTA	CTC	TTT	TTC	TTC	TCT	TCC	ATT	TTT	TTC	GTG	AGC	AAA	TGT
M	K	A	L	L	F	L	F	S	F	I	F	F	V	T	K	C
CAA	TGT	GAA	ACA	GAA	AGT	TAT	AAG	CAG	CTT	GTA	GCC	AAC	GTG	GAC	GAA	TTC
Q	C	E	T	E	S	Y	K	Q	L	V	A	N	V	D	E	F
AAC	ATC	TCG	CAG	CAC	CAA	TGC	GTG	AAA	AAA	CAA	TGT	CCC	GAG	AAC	TCT	GGC
N	I	S	Q	H	Q	C	V	K	K	Q	C	P	E	N	S	G
TGT	TTC	AGA	CAC	TTG	GAC	GAG	AGA	GAG	GAG	TGT	AAA	TGT	CTG	CTG	AAC	TAC
C	F	R	H	L	D	E	R	E	E	C	K	C	L	L	N	Y
AAA	CAG	GAG	GGC	GAC	AAG	TGC	GTG	GAG	AAC	CCC	AAC	CCG	ACC	TGT	AAC	GAG
K	Q	E	G	D	K	C	V	E	N	P	N	P	T	C	N	E
AAC	AAC	GCC	GGC	TGT	GAC	GCA	GAC	GCC	AAA	TGC	ACC	GAG	GAG	GAC	TCG	GGC
N	N	G	G	C	D	A	D	A	K	C	T	E	E	D	S	G
AGC	AAC	GCC	AAG	AAA	ATC	ACG	TGT	GAG	TGT	ACC	AAA	CCC	GAC	TCG	TAC	CCG
S	N	G	K	K	I	T	C	E	C	T	K	P	D	S	Y	P
CTG	TTC	GAC	GGC	ATC	TTC	TGC	AGC	TAA	TAA							
L	F	D	G	I	F	C	S	*	*							

FIGURE 1C

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GAA ACA GAA AGT TAT AAG CAG CTT GTA GGC AAC GTG GAC GAA TTC
 E T E S Y K Q L V A N V D E F
 AAC ATC TCG CAG CAC CAA TGC GTG AAA AAA CAA TGT CCC GAG AAC TCT GGC
 N I S Q H Q C V K K Q C P E N S G
 TGT TTC AGA CAC TTG GAC GAG AGA GAG GAG TGT AAA TGT CTG CTG AAC TAC
 C F R H L D E R E E C K C L L N Y
 AAA CAG GAG GGC GAC AAG TGC GTG GAG AAC CCC AAC CCG ACC TGT AAC GAG
 K Q E G D K C V E N P N P T C N E
 AAC AAC GGC GGC TGT GAC GCA GAC GCC AAA TGC ACC GAG GAG GAC TCG GGC
 N N G G C D A D A K C T E E D S G
 AGC AAC GGC AAG AAA ATC ACG TGT GAG TGT ACC AAA CCC GAC TCG TAC CCG
 S N G K K I T C E C T K P D S Y P
 CTG TTC GAC GGC ATC TTC TGC AGC TAA TAA
 L F D G I F C S * *

FIGURE 1D

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FIGURE 2

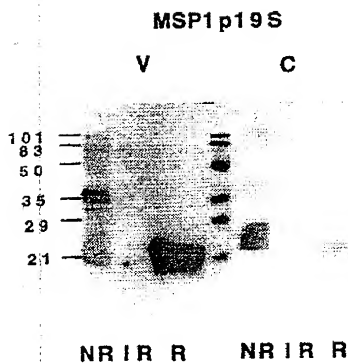


FIGURE 2B

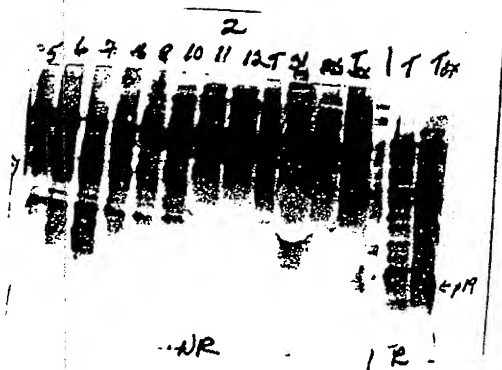


FIGURE 3

FIGURE 3B

[illegible]

	REGION III
161	<p>KEELONYPF LSSIOKEYST LVHKVSYTD TLKKI INNCO I EKKETETIV IRL EDSYKSK EEDLVYKSK KEDDYKSSG LEKLNKSL I KAELEKLPF INSKOIE/ES LVSKVHTYTD NLKVVNNCO LEKKAETV NKLODYNKD EKLEEVKSE KNEVKVSSG LEKLNKSL I KAELEKLPF INSKOIEYES LVKVNVTYD NLKVVNNCO LEKKAETV NKLODYNKD EKLEEVKSE KNEVKVSSG LEKLNKSL I K-EL-KYLPF L-S-QKEY-- LV-KV--YTD LKKI INNCO EKKKE-E-V-V -KL-DY-XMD E-L-YK-S-K--VKSSG LEKLN-SKL I</p>

271

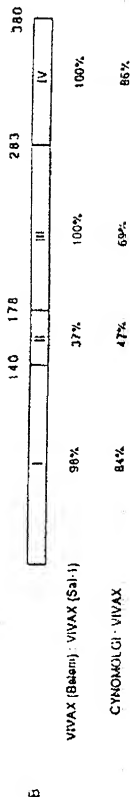
← ↑

WUESKKAALS ELUNVOTOML **PUTS**SEHRCIO INVPENAACY RYLDGTEWR CILLYFKEDAG KCVPAARRITC KOKNGGCAPE AECKMUND-
KEREKEKLS OLUNVOTOLL **THS**SENTCIO INVPONAACY RYLDGTEWR CILLYFKEEGG KCVPASNVTC KDNNGGCAPE AECKMIDSNK
KEREKEKLS OLUNVOTOLL **THS**SENTCIO INVPONAACY RYLDGTEWR CILLYFKEEGG KCVPASNVTC KDNNGGCAPE AECKMIDSNK
...ESK...LS LUNVOTO-L -WSEK-CID THVP-NAACY RYLDGTEWR CILLYFKEEGG KCVPA-N-TC KO-JGGGCAPE AECKM-ID-N.

Cynwadi
... (Bakem)
... msa(f)
C-ynwadi

[illegible]

FIGURE 4



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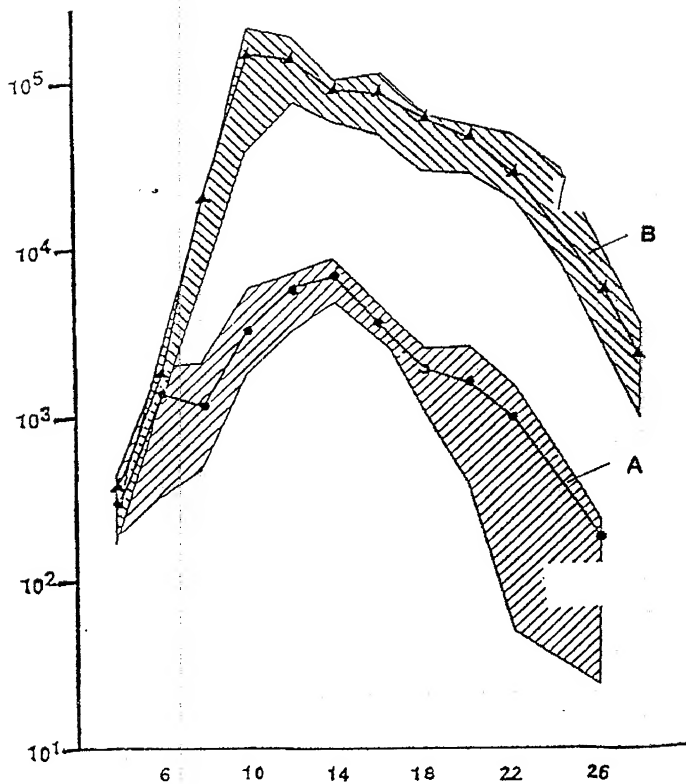


FIGURE 5

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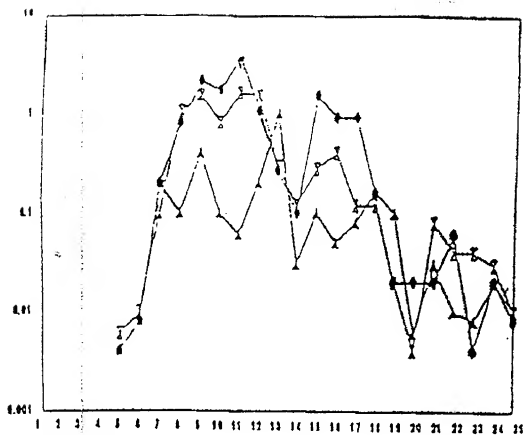


FIGURE 6A

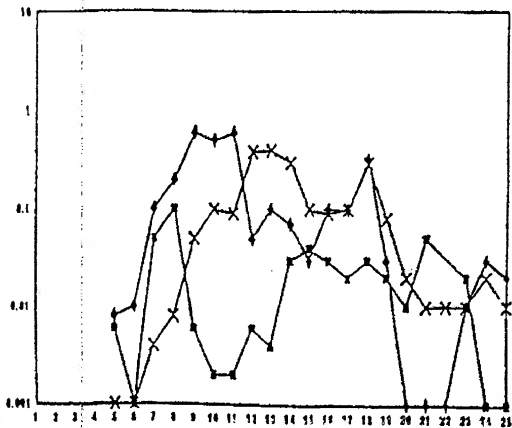


FIGURE 6B

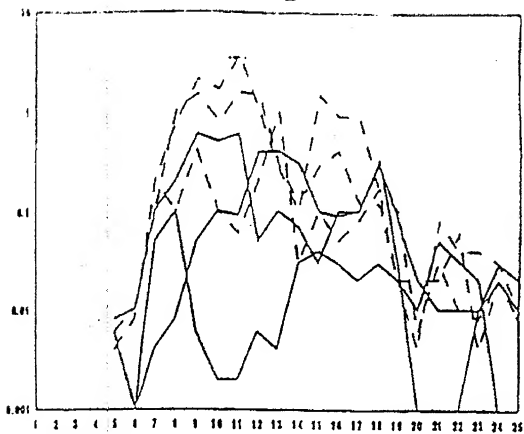


FIGURE 6C

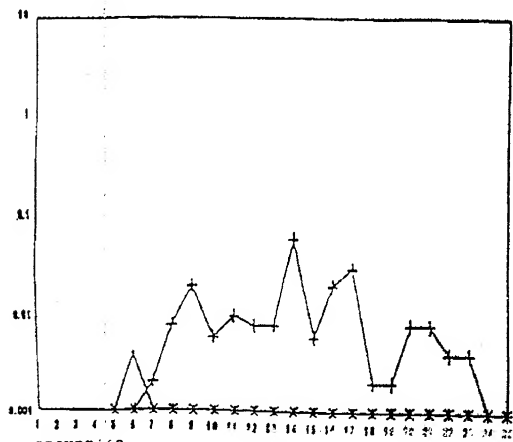


FIGURE 6D

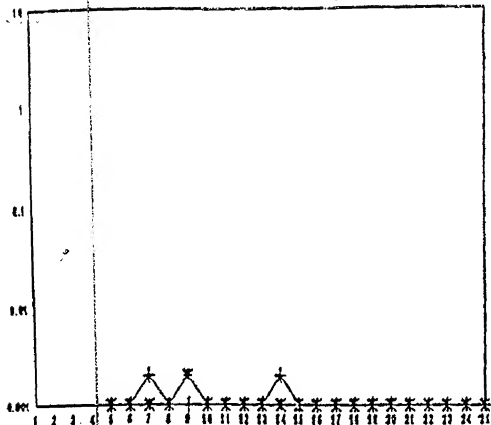


FIGURE 6E

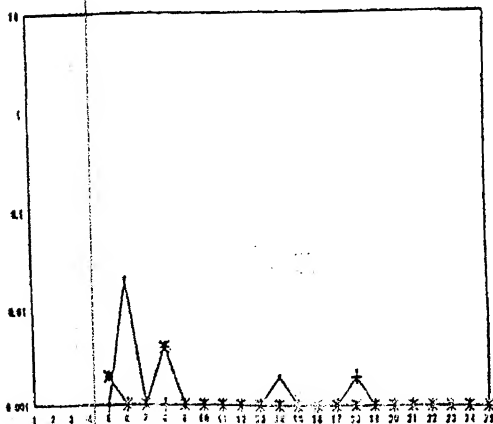


FIGURE 6F

Vaccination test: recombinant MSP-1 (p42 and p19) from *Plasmodium cynomolgi* in the *Macaca sinica* (toque mac

Year	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2423	2424	2425	2426	2427	2428	2429	2430	2431	2432	2433	2434	2435	2436	2437	2438	2439	2440	2441	2442	2443	2444	2445	2446	2447	2448	2449	2450	2451	2452	2453	2454	2455	2456	2457	2458	2459	2460	2461	2462	2463	2464	2465	2466	2467	2468	2469	2470	2471	2472	2473	2474	2475	2476	2477	2478	2479	2480	2481	2482	2483	2484	2485	2486	2487	2488	2489	2490	2491	2492	2493	2494	2495	2496	2497	2498	2499	2500	2501	2502	2503	2504	2505	2506	2507	2508	2509	2510	2511	2512	2513	2514	2515	2516	2517	2518	2519	2520	2521	2522	2523	2524	2525	2526	2527	2528	2529	2530	2531	2532	2533	2534	2535	2536	2537	2538	2539	2540	2541	2542	2543	2544	2545	2546	2547	2548	2549	2550	2551	2552	2553	2554	2555	2556	2557	2558	2559	2560	2561	2562	2563	2564	2565	2566	2567	2568	2569	2570	2571	2572	2573	2574	2575	2576	2577	2578	2579	2580	2581	2582	2583	2584	2585	2586	2587	2588	2589	2590	2591	2592	2593	2594	2595	2596	2597	2598	2599	2600	2601	2602	2603	2604	2605	2606	2607	2608	2609	2610	2611	2612	2613	2614	2615	2616	2617	2618	2619	2620	2621	2622	2623	2624	2625	2626	2627	2628	2629	2630	2631	2632	2633	2634	2635	2636	2637	2638	2639	2640	2641	2642	2643	2644	2645	2646	2647	2648	2649	2650	2651	2652	2653	2654	2655	2656	2657	2658	2659	2660	2661	2662	2663	2664	2665	2666	2667	2668	2669	2670	2671	2672	2673	2674	2675	2676	2677	2678	2679	2680	2681	2682	2683	2684	2685	2686	2687	2688	2689	2690	2691	2692	2693	2694	2695	2696	2697	2698	2699	2700	2701	2702	2703	2704	2705	2706	2707	2708	2709	2710	2711	2712	2713	2714	2715	2716	2717	2718	2719	2720	2721	2722	2723	2724	2725	2726	2727	2728	2729	2730	2731	2732	2733	2734	2735	2736	2737	2738	2739	2740	2741	2742	2743	2744	2745	2746	2747	2748	2749	2750	2751	2752	2753	2754	2755	2756	2757	2758	2759	2760	2761	2762	2763	2764	2765	2766	2767	2768	2769	2770	2771	2772	2773	2774	2775	2776	2777	2778	2779	2780	2781	2782	2783	2784	2785	2786	2787	2788	2789	2790	2791	2792	2793	2794	2795	2796	2797	2798	2799	2800	2801	2802	2803	2804	2805	2806	2807	2808	2809	2810	2811	2812	2813	2814	2815	2816	2817	2818	2819	2820	2821	2822	2823	2824	2825	2826	2827	2828	2829	2830	2831	2832	2833	2834	2835	2836	2837	2838	2839	2840	2841	2842	2843	2844	2845	2846	2847	2848	2849	2850	2851	2852	2853	2854	2855	2856	2857	2858	2859	2860	2861	2862	2863	2864	2865	2866	2867	2868	2869	2870	2871	2872	2873	2874	2875	2876	2877	2878	2879	2880	2881	2882	2883	2884	2885	2886	2887	2888	2889	2890	2891	2892	2893	2894	2895	2896	2897	2898	2899	2900	2901	2902	2903	2904	2905	2906	2907	2908	2909	2910	2911	2912	2913	2914	2915	2916	2917	2918	2919	2920	2921	2922	2923	2924	2925	2926	2927	2928	2929	2930	2931	2932	2933	2934	2935	2936	2937	2938	2939	2940	2941	2942	2943	2944	2945	2946	2947	2948	2949	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— = Absence of parasites in 400 microscopic fields

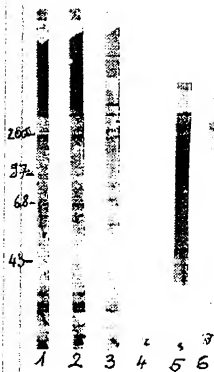


FIGURE 7

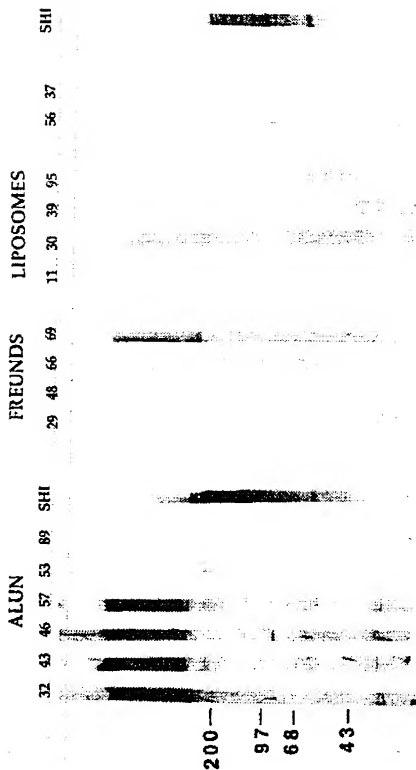
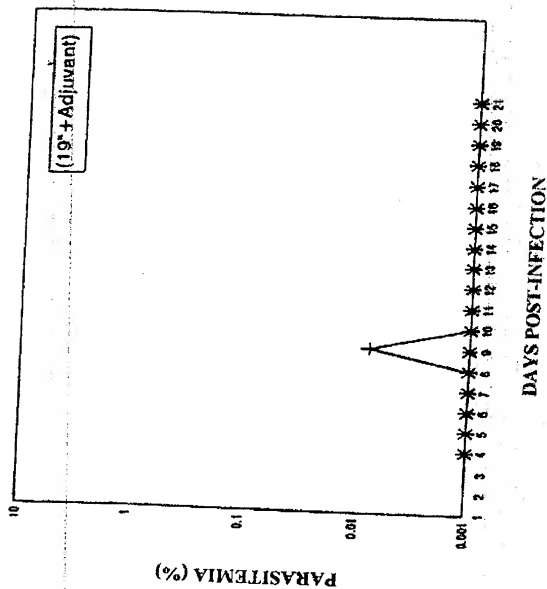


FIGURE 7B

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque
macaque; second challenge infection



Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque; second challenge

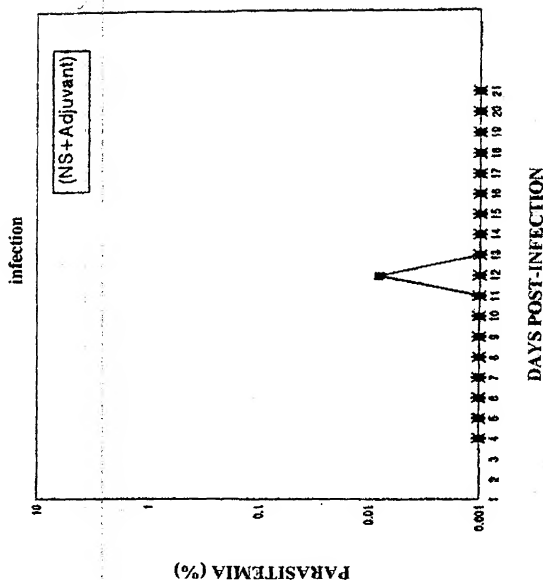
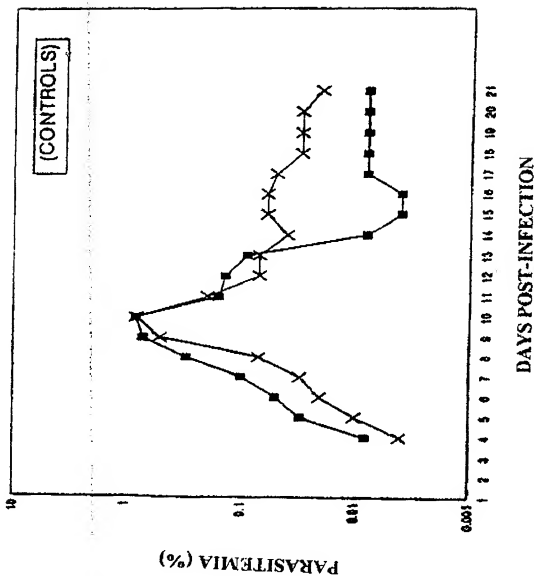


FIGURE 8B

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque; second challenge infection

FIGURE 8C



Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the
Macaca sinica toque macaque; second challenge infection

Year	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96
Month	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Days post-infection	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	20
Days after challenge	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Vaccination p19																			
T 426	-	-	-	-	-	.008	-	-	-	-	-	-	-	-	-	-	-	-	-
T 427	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T 429	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls																			
Physiological water																			
FCA/FIA																			
T 436	-	-	-	-	-	-	-	-	.008	-	-	-	-	-	-	-	-	-	-
T 425	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T 438	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls																			
Non vaccinated																			
T 448	.008	.03	.05	0.1	0.3	0.7	0.8	.16	.14	.09	.008	.004	.004	.008	.008	.008	.008	.008	.008
T 449	.004	.01	.02	.03	0.07	0.5	0.8	0.2	.07	.07	.04	.06	.06	.05	.03	.03	.03	.03	.02

- = Absence of parasites in 400 microscopic fields

FIGURE 8D

FIGURE 9A

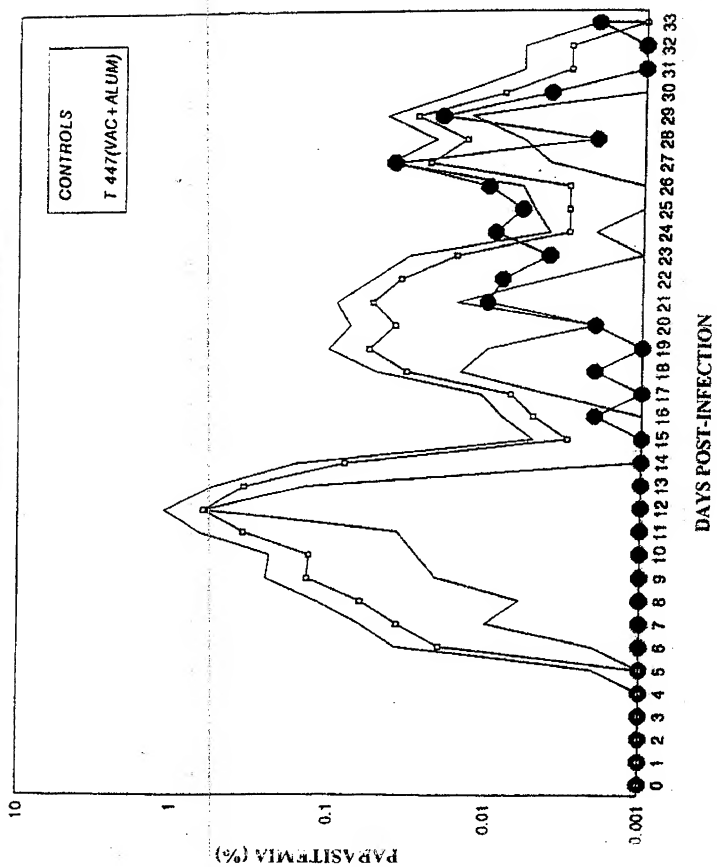


FIGURE 9B

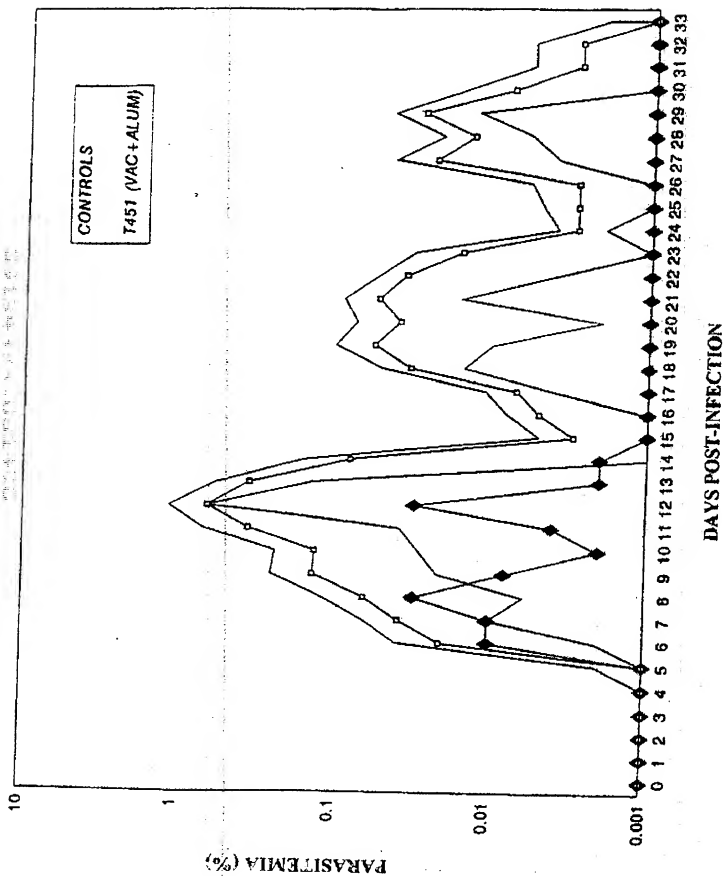


FIGURE 9C

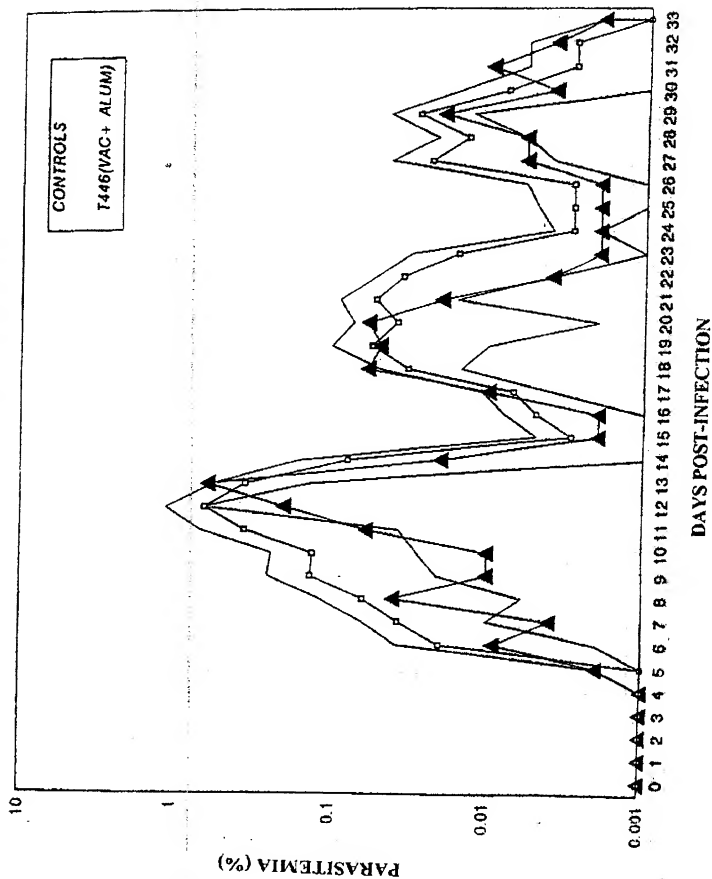
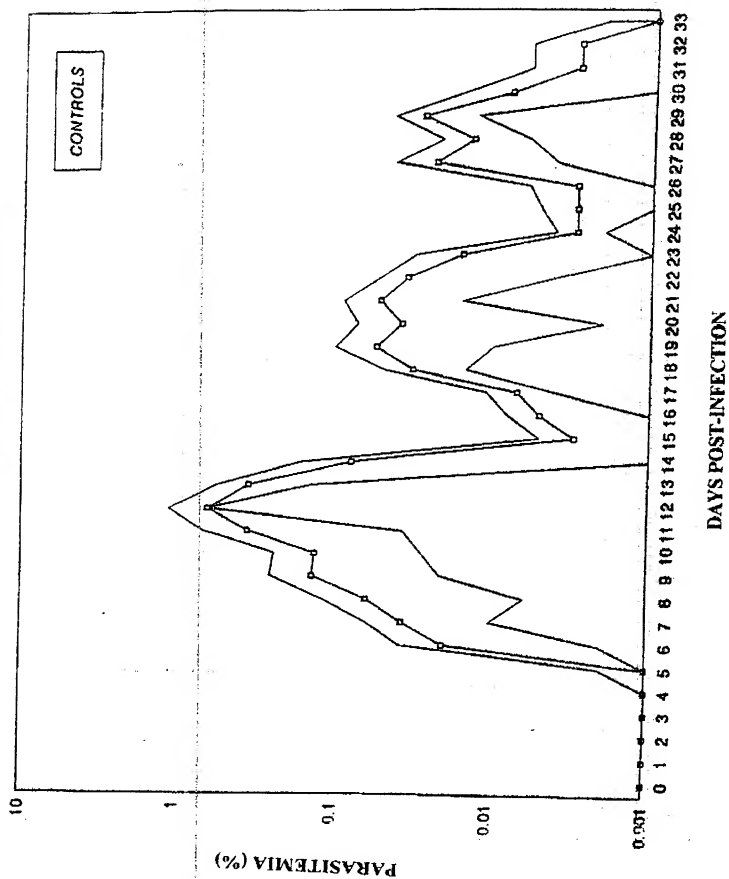


FIGURE 90



cynomolgi in alom

FIGURE 9E

— negative for parasites in 400 microscopic fields

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI

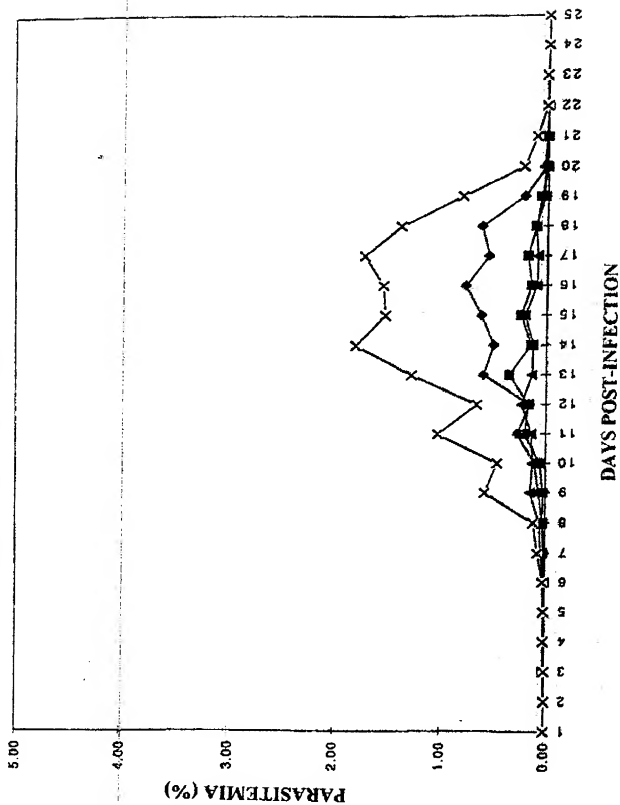
SCIUREUS SQUIRREL MONKEY

MSP-1 p19 VACCINATION WITH ALUM

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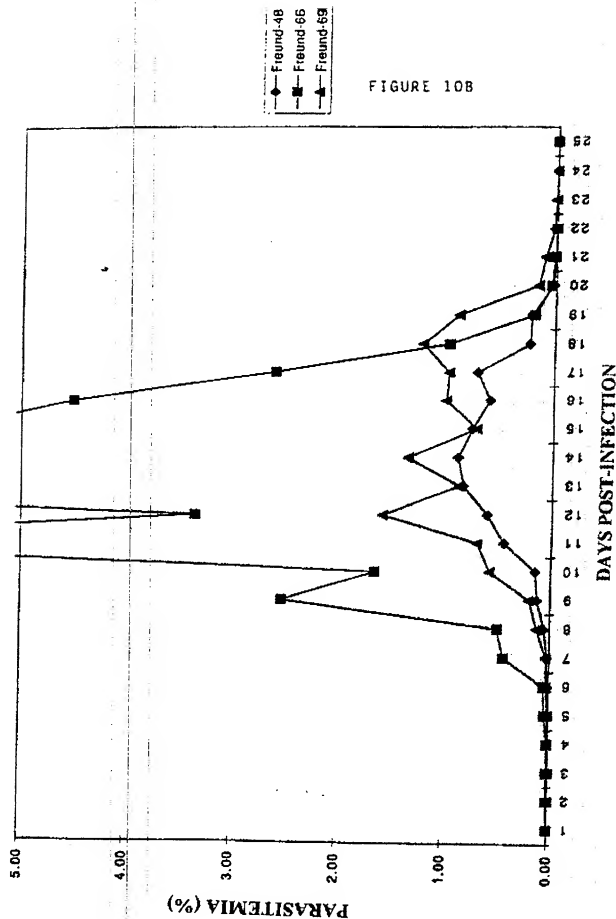


FIGURE 10A



PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI
 SCITREUS SQUIRREL MONKEY

MSP-1 p19 VACCINATION WITH FREUNDS



PLASMODIUM FALCIPARUM VACCINATION TEST IN *SAIMIRI*

SCITUREUS SQUIRREL MONKEY

MSP-1 p19 VACCINATION WITH LIPOSOMES

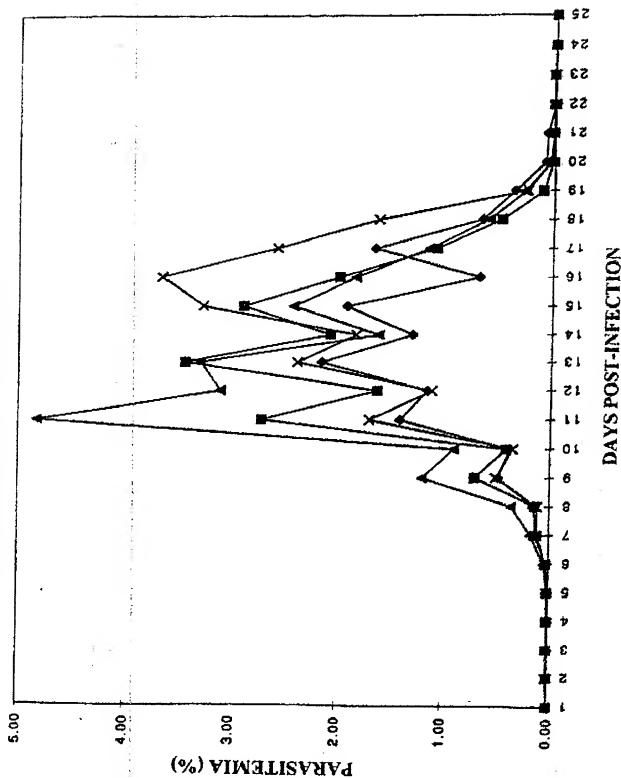
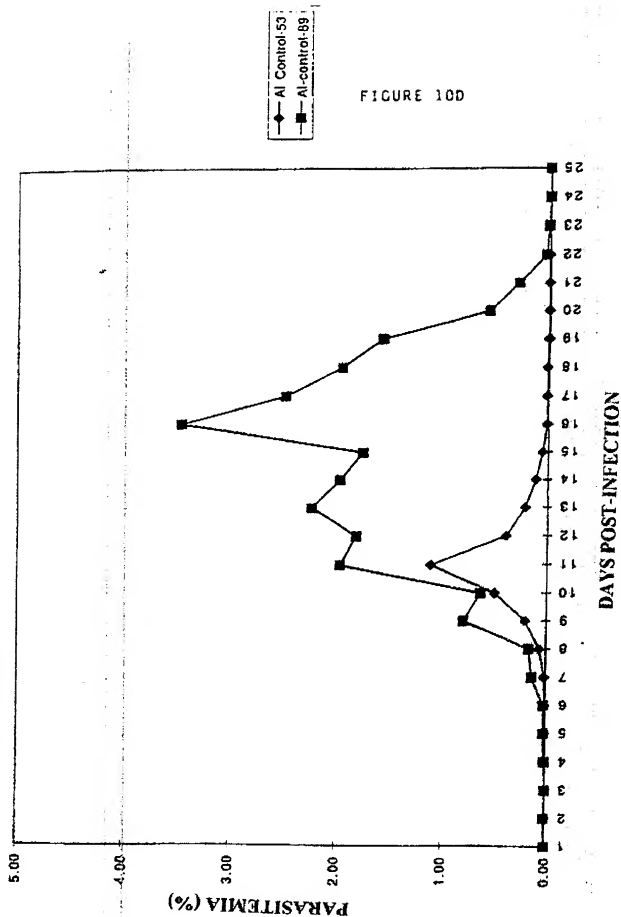


FIGURE 10C

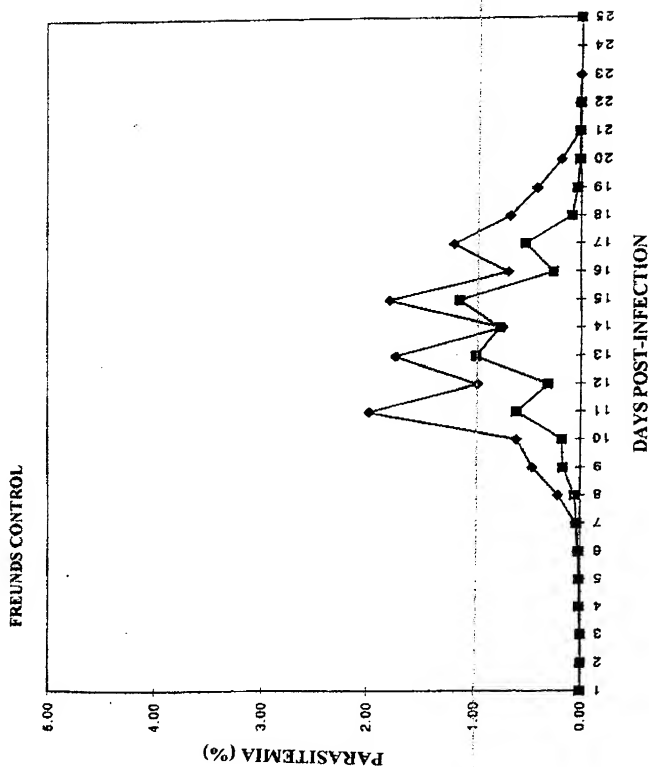
PLASMODIUM FALCIPARUM VACCINATION TEST IN SAMIRI
SCIUREUS SQUIRREL MONKEY
ALUM CONTROL

FIGURE 10D



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FIGURE 10E



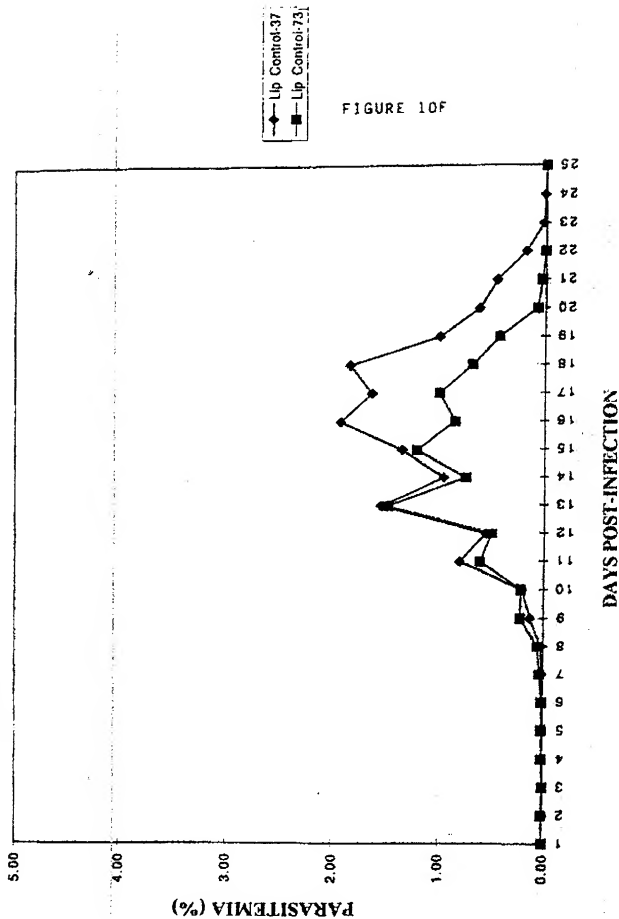
PLASMODIUM FALCIPARUM VACCINATION TEST IN SAMIRI

SCIUREUS SQUIRREL MONKEY

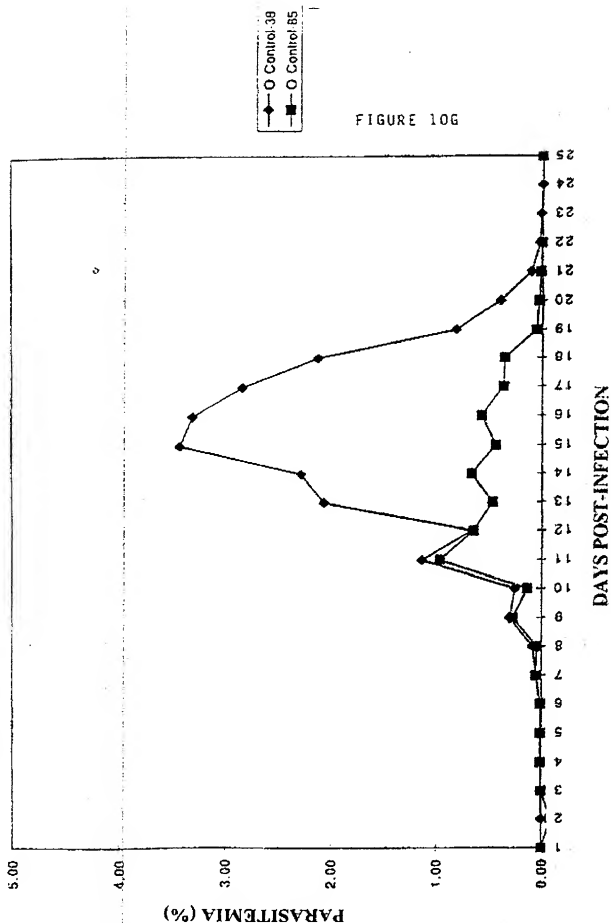
LIPOSOME CONTROL

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FIGURE 10F



PLASMODIUM FALCIPARUM VACCINATION TEST IN SAMIRI
SCIUREUS SQUIRREL MONKEY
PHYSIOLOGICAL WATER CONTROL



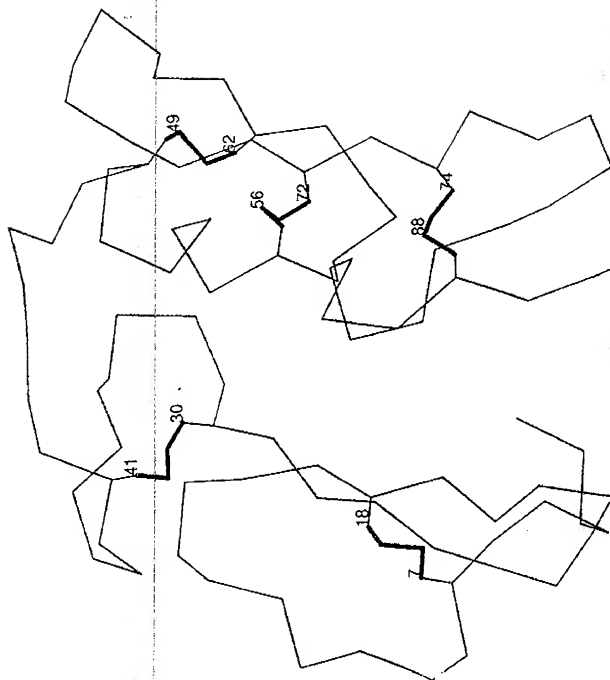


Figure 11A: Backbone of MSP1(19) showing disulfide bridges in bold line

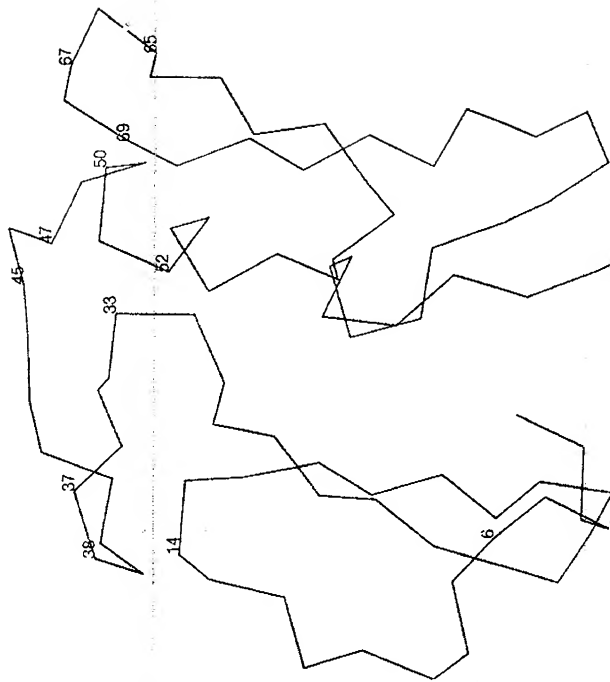


Figure 11B: Backbone of MSP1(19) showing positions of sequence differences between *P. cynomolgi* and *P. vivax*

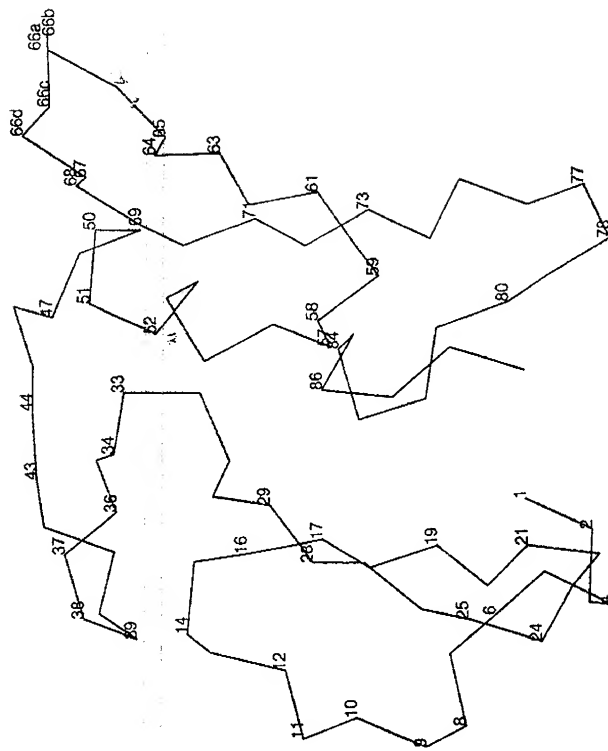
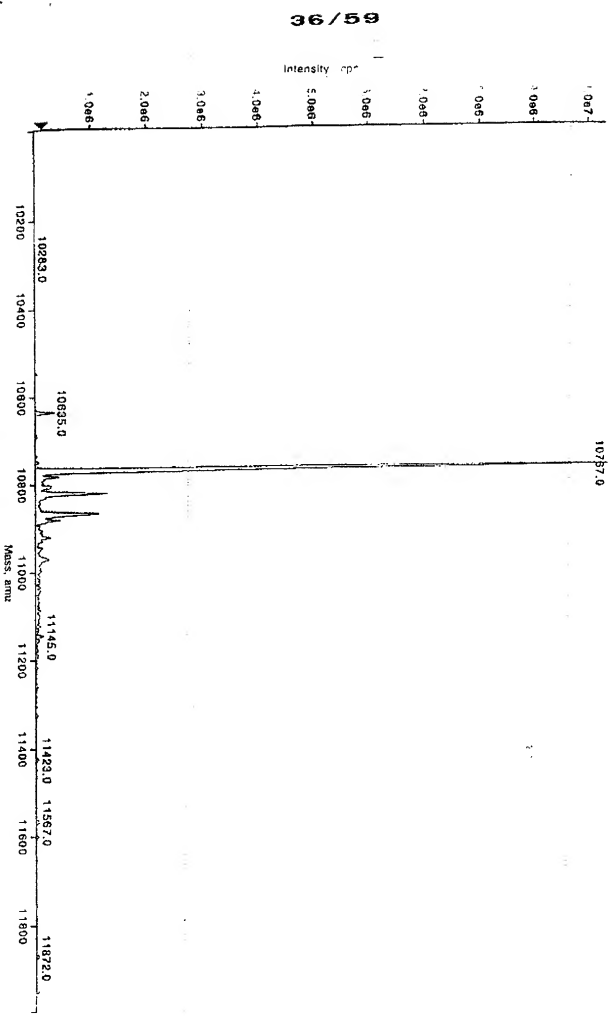


Figure 11C: Backbone of homology-modelled MSP1(19) of *P. falciparum* with positions of sequence differences with *P. cynomolgi*

U

FIGURE 12A (RECONSTRUCTED MASS SPECTRA P. CYNOMOLGB)



1.03e7 cps

FIGURE 12B (m/z SPECTRA, P. CYNOLOGI)

2. 1. 5ps

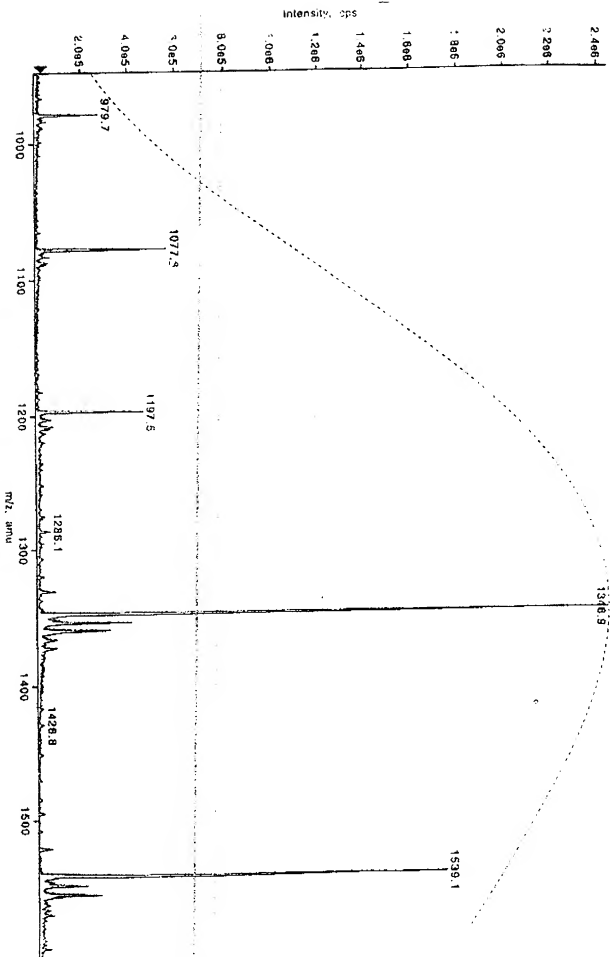
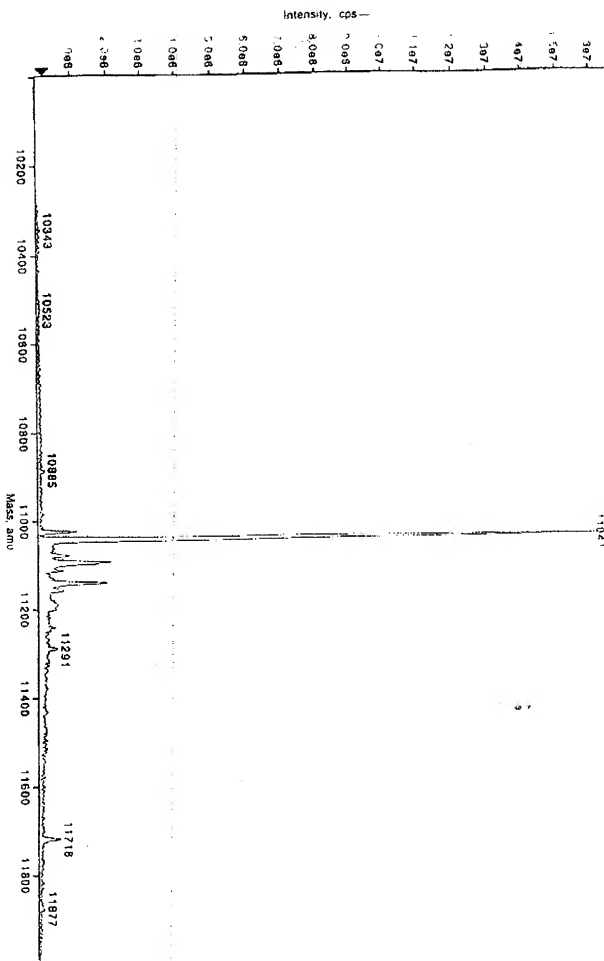
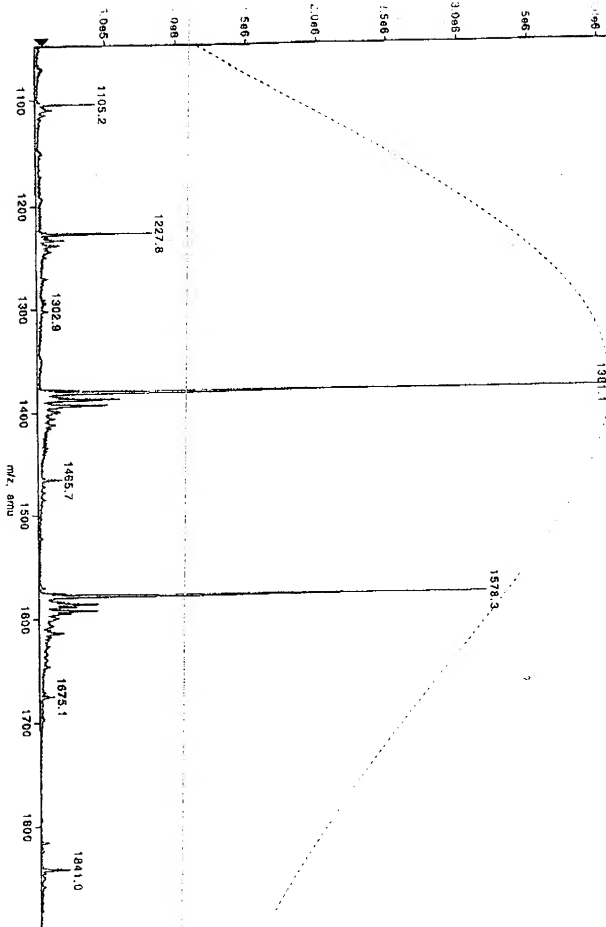


FIGURE 12C (RECONSTRUCTED MASS SPECTRA P. FLACPARUM)



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Intensity, cps

FIGURE 12D (m/z SPECTRA P. TALCIPARUM)

A 0766 cps

% Intensity

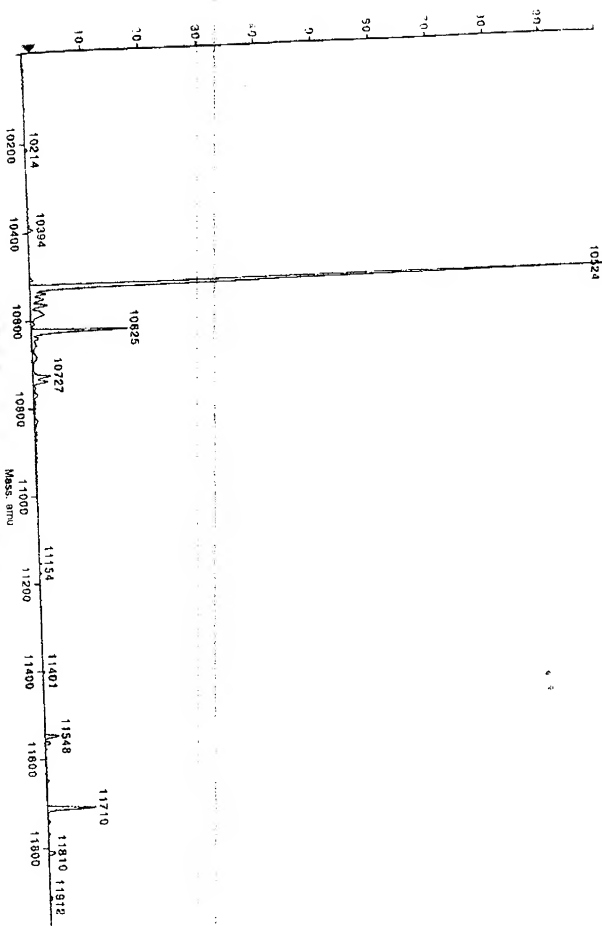
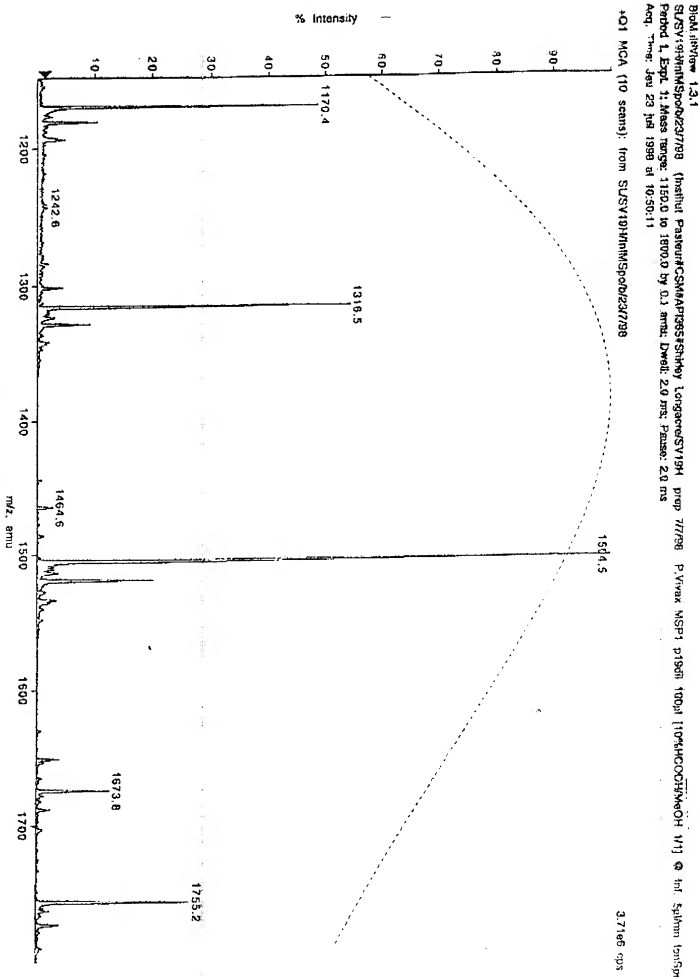


FIGURE 12E RECONSTRUCTED MASS SPECTRA P. VIVAXO

FIGURE 12F (m/z SPECTRA P. VIVAX)



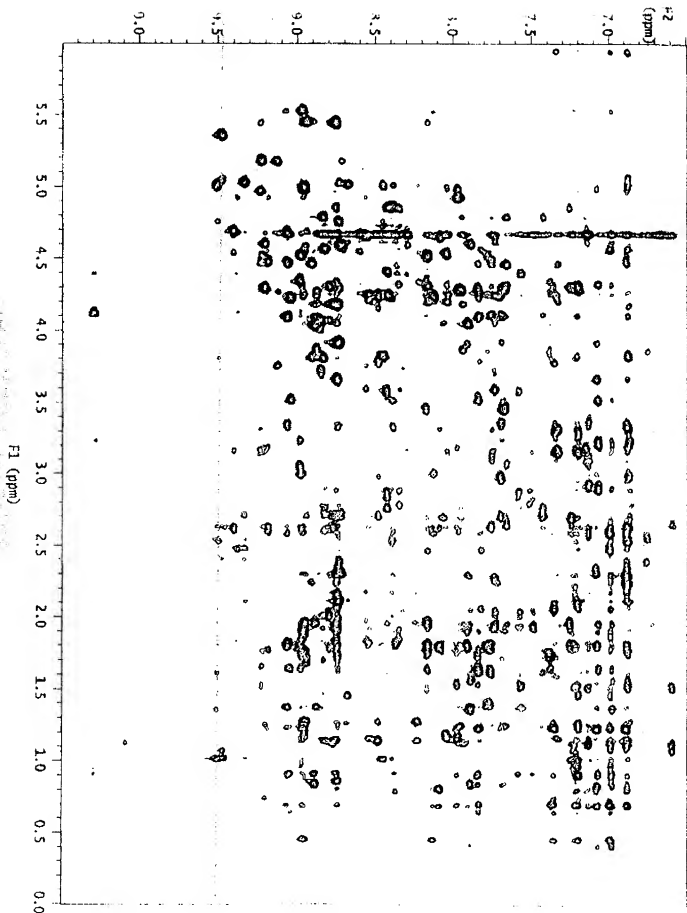


FIGURE 12.0a (NOESTY CYNOMOLGIN)

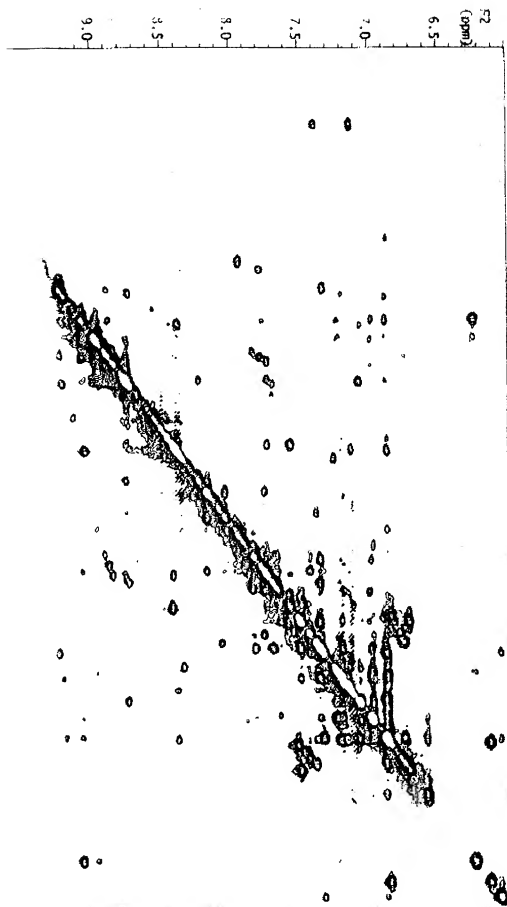
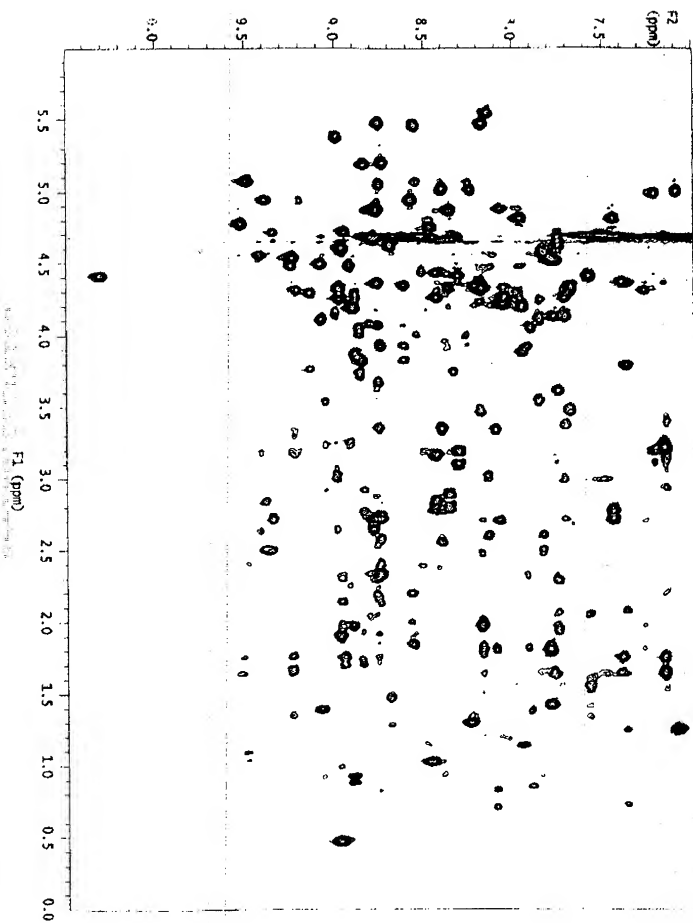


FIGURE 12.0b (WEST CYTOMOLOG)

FIGURE 12.6C (TOXSY CYANOMOLG1)



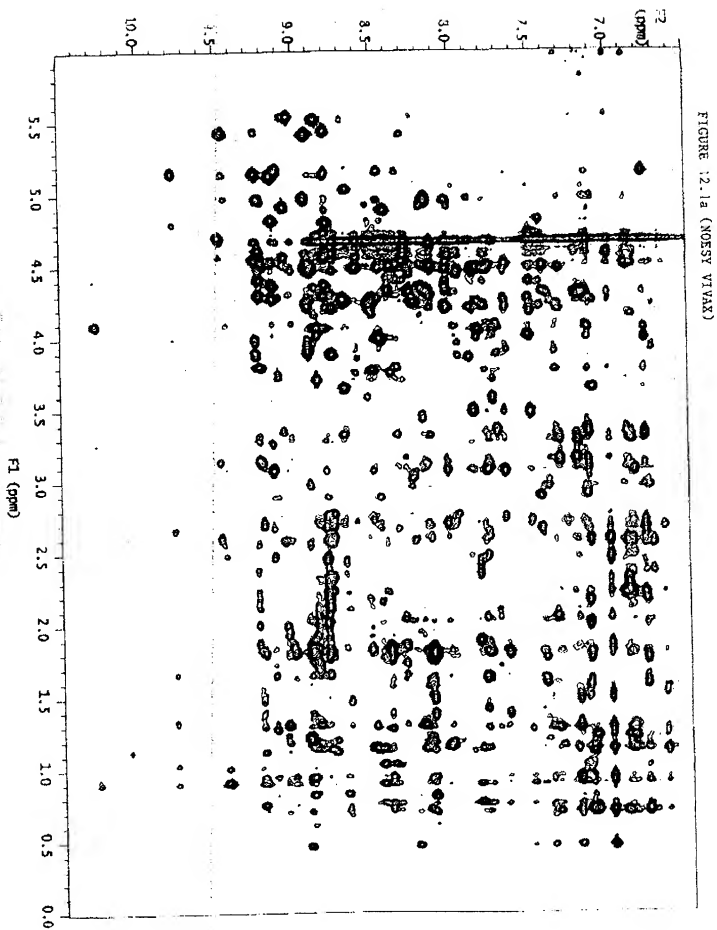


FIGURE 12.1b (NOESY VIVAX)

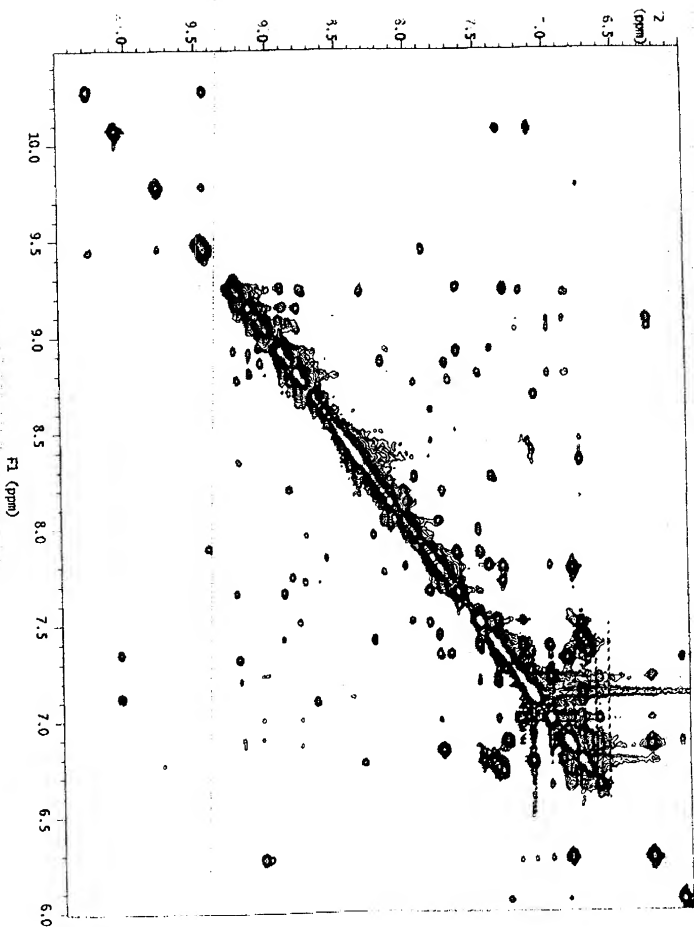


FIGURE 12.1c (TOCST VIVAX)

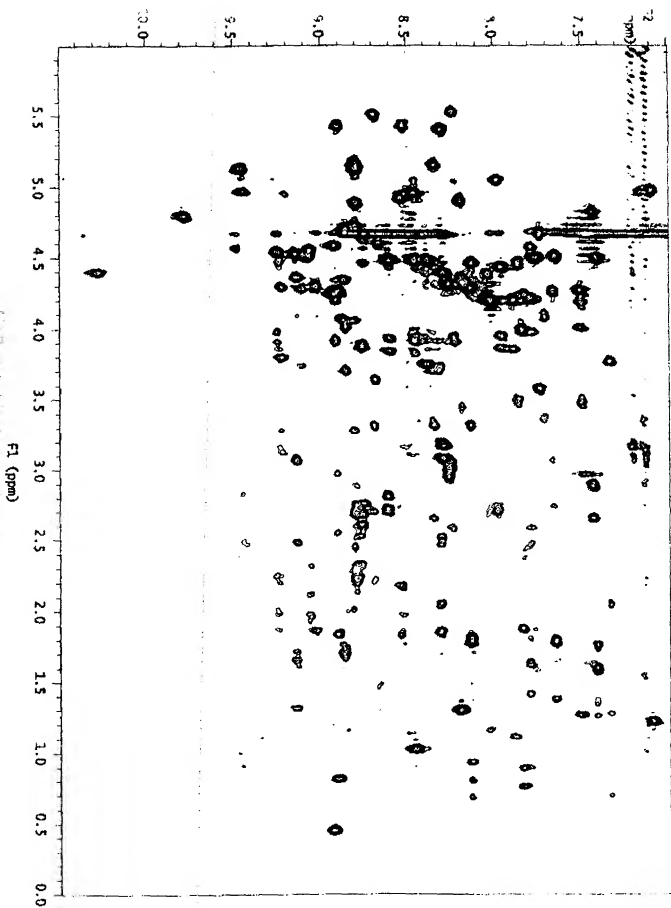


FIGURE 12.2a (NOESY FALCIPARUM)

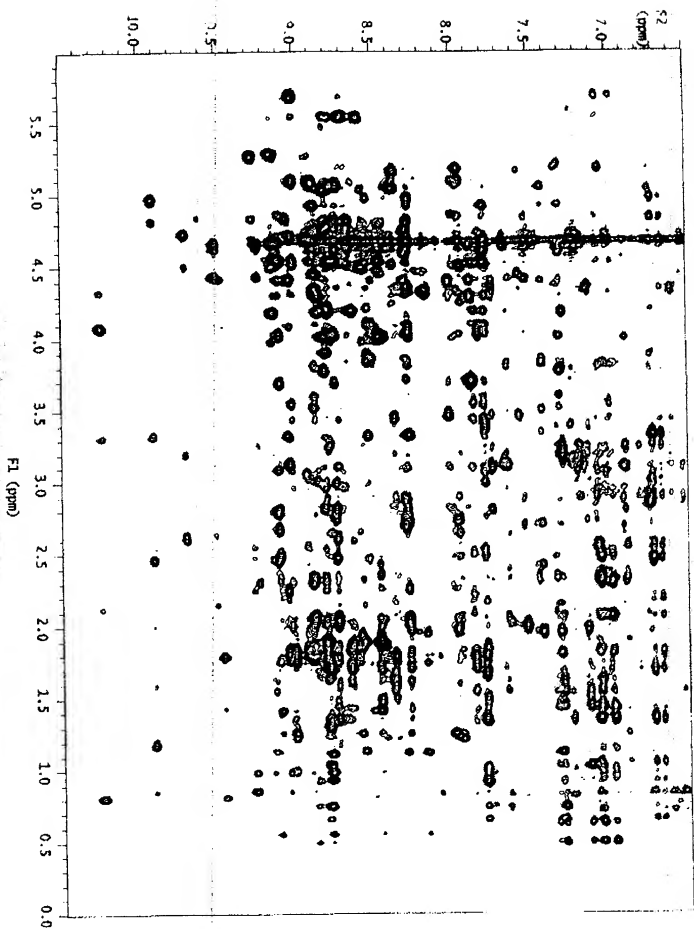


FIGURE 12.2b (NOESY FALCIPARUM)

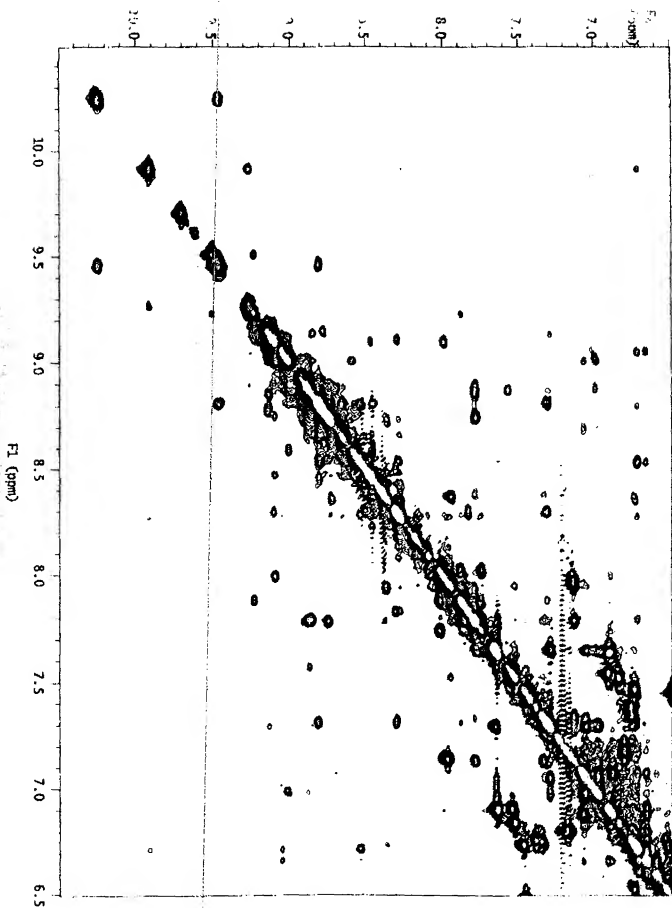
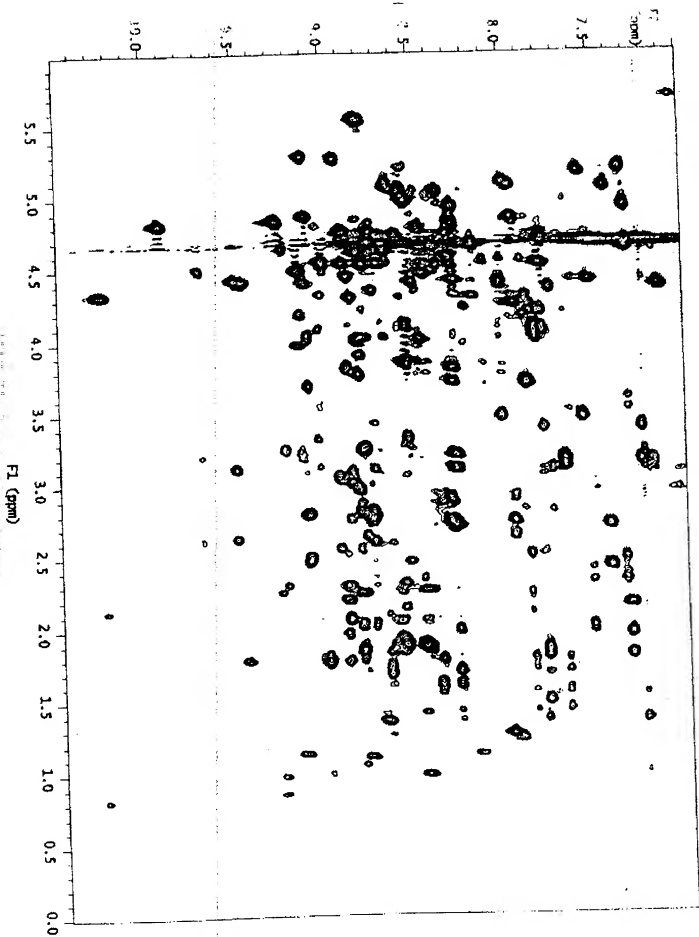


FIGURE 12.2c (TOSCY FALCIPARUM)



Monkey 426 anti-MSP1_{p 19}

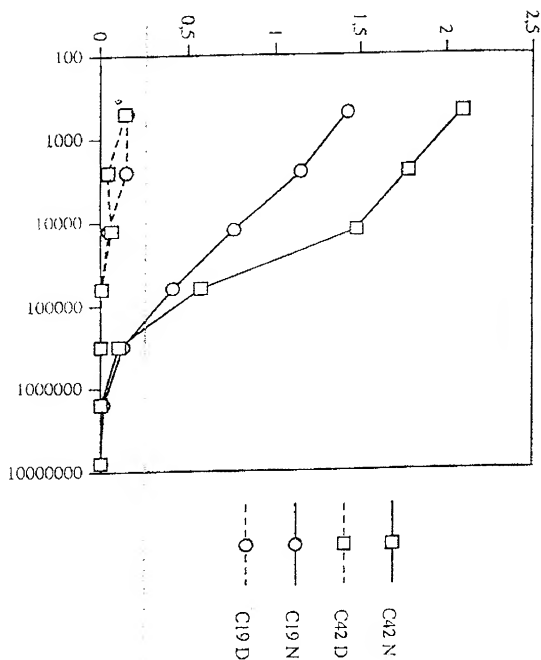


FIGURE 13A

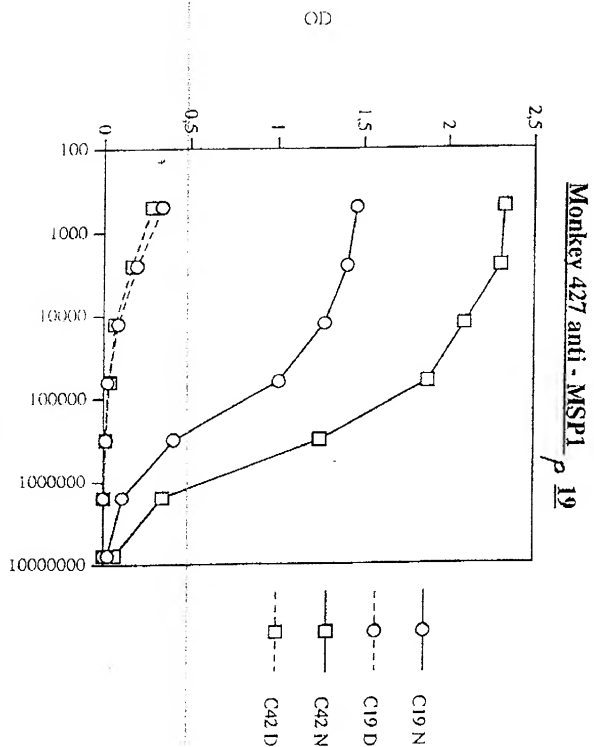


FIGURE 13B

CIC

Monkey 429 anti - MSP1 p 19

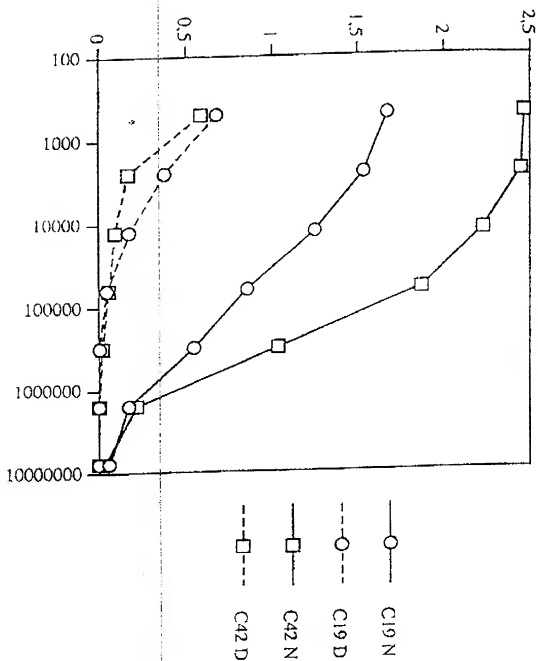


FIGURE 13C

Monkey 428 anti - MSP1_{p42}

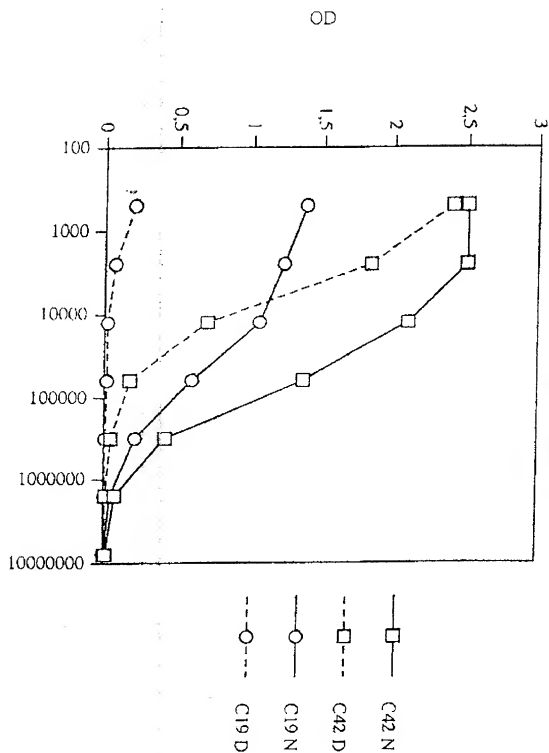


FIGURE 13D

Monkey 434 anti - MSP1
p 42

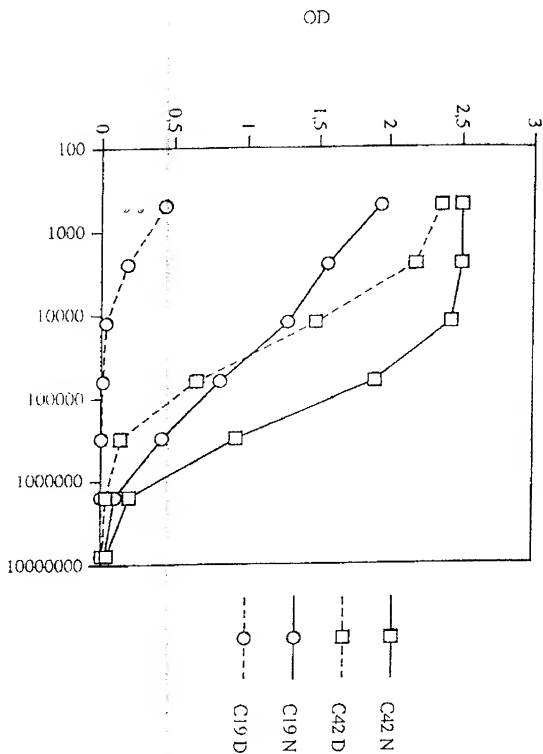
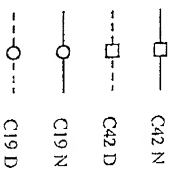


FIGURE 13E

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[illegible]

A

Endemic human antisera reactivity with recombinant *Plasmodium vivax* MSP1₁₉
(immunoaffinity purified)

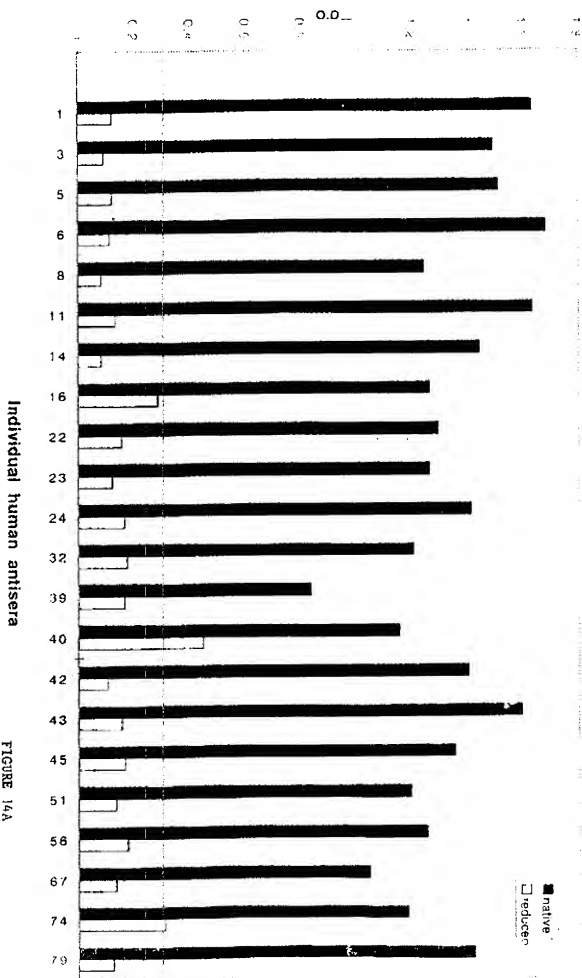
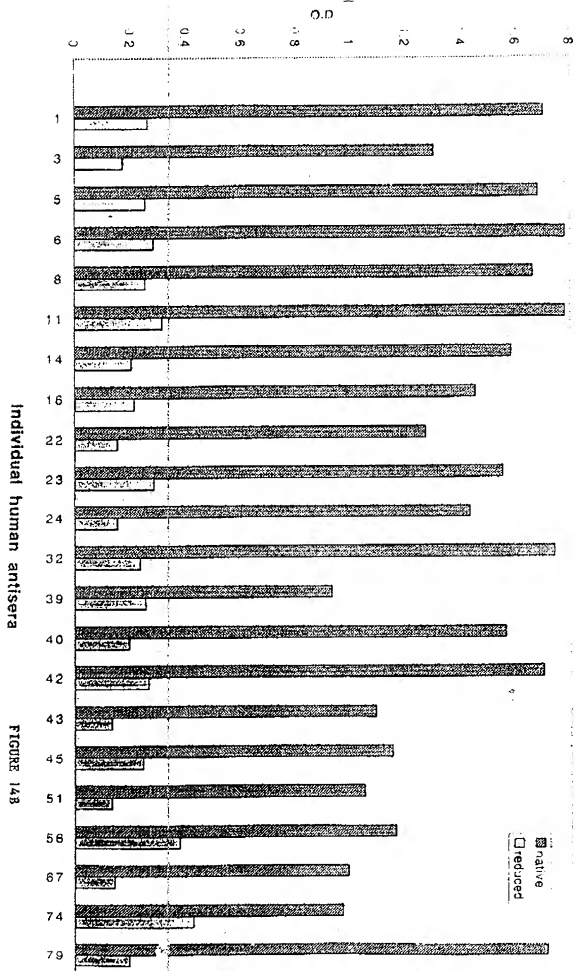


FIGURE 14A

Endemic human antisera reactivity with recombinant *Plasmodium vivax* MSP1₁₉
(metalloaffinity purified)

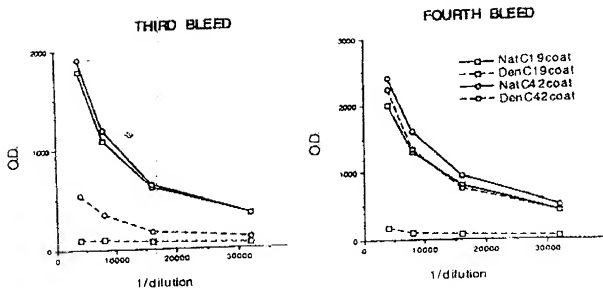


Individual human antisera

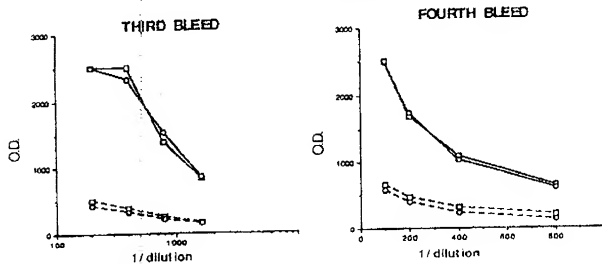
FIGURE 148

FIGURE 15

ANTI-C42 RESPONSE

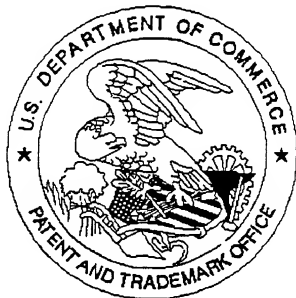


ANTI-C19 RESPONSE



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